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MODIFIED *FUSARIUM* MYCOTOXINS: A THREAT IN DISGUISE?

ALEXIS V. NATHANAIL

DOCTORAL DISSERTATION

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ABSTRACT

Fungi are capable of producing an array of heterogenous toxic secondary metabolites, i.e. mycotoxins, which may acutely or chronically impact human and animal health following the consumption of contaminated agricultural commodities. Mycotoxins, like most xenobiotics, are prone to structural alterations via metabolic processes in living organisms but can also undergo changes during food manufacturing. The resulting compounds, defined as "modified mycotoxins", possess distinct chemical properties, with potentially unique toxicological characteristics, and often coexist with their precursor forms in food- and feedstuffs.

The impetus of this Ph.D. thesis largely stems from the dearth of evidence available on these compounds and aspires to contribute evidence for addressing the underlying debate: Are modified mycotoxins relevant to food/feed safety?

In this context, liquid chromatography–tandem mass spectrometric methods, employing fit-for-purpose sample preparation approaches, were developed and validated for the simultaneous determination of *Fusarium* mycotoxins and their modified forms. At first, conventional sample preparation techniques commonly utilised in mycotoxin analyses were evaluated against automated on-line sample clean-up. On-line clean-up and the standard "extract and shoot" approach offered optimal overall performance and achieved compliance with legislative criteria. The natural occurrence of the *Fusarium* mycotoxins HT-2 toxin, T-2 toxin, deoxynivalenol, nivalenol, zearalenone and derivatives thereof was investigated by conducting a nationwide survey of Finnish barley, oats and wheat grains. Deoxynivalenol was the most abundant mycotoxin (in 93% of the cereal samples), and at unusually high levels compared to adjacent years, followed by the modified mycotoxin deoxynivalenol-3-glucoside (81%). All 10 additional modified mycotoxins included in the method were detectable at widely varying concentrations. The relative proportions of modified/parent mycotoxins were mostly between 15–55%.

Furthermore, the metabolism of HT-2 toxin and T-2 toxin was studied in barley and wheat. Specifically, tracing of their metabolism was accomplished by untargeted metabolomics based on stable isotopic labelling and liquid chromatography–high resolution mass spectrometry. Structural elucidation of the detected compounds indicated the presence of several novel modified mycotoxins, including glucoside, malonyl-glucoside, acetyl and feruloyl conjugates of the parent toxins. Time course kinetics of the *in planta* metabolites revealed the HT-2 toxin-3-glucoside as the primary detoxification product, which was rapidly formed in both crops. The experiments also determined the extent of metabolism of the parent toxins, while highlighting those modified forms present at harvest. Lastly, the metabolic fate of HT-2 toxin, T-2 toxin, deoxynivalenol and deoxynivalenol-3-glucoside was investigated during a four-day beer brewing fermentation with lager yeast. Yeast tolerated high toxin levels and was able to remove 9–34% of dosed toxins from wort by adsorption and/or biotransformation.

The original contribution of this work can be summarised as the discovery of several novel modified *Fusarium* mycotoxins and related metabolic pathways, generation of essential natural occurrence data and gaining of further insight into mycotoxin-plant/fungal interactions, all of which were facilitated by state-of-the-art analytical tools.

PREFACE

This doctoral thesis was part of the Myco-DETECT project (No. 401/31/2011), which was a collaboration between the Finnish Food Authority (Ruokavirasto; previously Evira), the VTT Technical Research Centre of Finland, the Finnish Institute for Verification of the Chemical Weapons Convention (VERIFIN), the Finnish Customs Laboratory, Thermo Fisher Scientific and the Department of Agrobiotechnology, IFA-Tulln, University of Natural Resources and Life Sciences, Vienna (BOKU), Austria.

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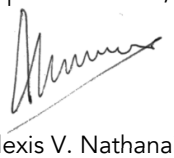

Alexis V. Nathanail

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to throughout the text by their Roman numerals I–V:

I. Nathanail AV, Sarikaya E, Jestoi M, Godula M, Peltonen K. 2014. Determination of deoxynivalenol and deoxynivalenol-3-glucoside in wheat and barley using liquid chromatography coupled to mass spectrometry: On-line clean-up *versus* conventional sample preparation techniques. Journal of Chromatography A 1374: 31-39.

II. Nathanail AV, Syvähuoko J, Malachová A, Jestoi M, Varga E, Michlmayr H, Adam G, Sieviläinen E, Berthiller F, Peltonen K. 2015. Simultaneous determination of major type A and B trichothecenes, zearalenone and certain modified metabolites in Finnish cereal grains with a novel liquid chromatography-tandem mass spectrometric method. Analytical Bioanalytical Chemistry 407: 4745-4755.

III. Meng-Reiterer J, Varga E, Nathanail AV, Bueschl C, Rechthaler J, McCormick SP, Michlmayr H, Malachová A, Fruhmann P, Adam G, Berthiller F, Lemmens M, Schuhmacher R. 2015. Tracing the metabolism of HT-2 toxin and T-2 toxin in barley by isotope-assisted untargeted screening and quantitative LC–HRMS analysis. Analytical Bioanalytical Chemistry 407: 8019-8033.

IV. Nathanail AV, Varga E, Meng-Reiterer J, Bueschl C, Michlmayr H, Malachová A, Fruhmann P, Jestoi M, Peltonen K, Adam G, Lemmens M, Schuhmacher R, Berthiller F. 2015. Metabolism of the *Fusarium* mycotoxins T-2 toxin and HT-2 toxin in wheat. Journal of Agricultural and Food Chemistry 63 (35): 7862-7872.

V. Nathanail AV, Gibson B, Han L, Peltonen K, Ollilainen V, Jestoi M, Laitila A. 2016. The lager yeast *Saccharomyces pastorianus* removes and transforms *Fusarium* trichothecene mycotoxins during fermentation of brewer's wort. Food Chemistry 203: 448-455.

AUTHOR CONTRIBUTION TO PAPERS I–V

I. Alexis V. Nathanail, M.Sc., planned the study and prepared the manuscript in collaboration with the other co-authors. He also performed the experimental work, including sample preparation, LC–ESI–TQ–MS analysis and interpretation of the results. Alexis V. Nathanail was the corresponding author of this article.

II. Alexis V. Nathanail planned the study, interpreted the results and prepared the manuscript in collaboration with the other co-authors. Jenna Syvähuoko, M.Sc., carried out the majority of the LC–ESI–TQ–MS analysis under the supervision of Alexis V. Nathanail, who was also the corresponding author of this article.

III. Alexis V. Nathanail planned the study in collaboration with the other co-authors and performed part of the greenhouse experiments and LC–HRMS analysis. Alexis V. Nathanail participated in the interpretation of the results and preparation of the manuscript.

IV. Alexis V. Nathanail planned the study and prepared the manuscript in collaboration with the other co-authors. Alexis V. Nathanail and Dr Elisabeth Varga had an equal contribution to the experimental work and the interpretation of the results and were the primary authors of the article.

V. Alexis V. Nathanail planned the study, interpreted the results, performed LC–ESI–QTOF–MS analysis and prepared the manuscript in collaboration with the other co-authors. Li Han, M.Sc., carried out the majority of the LC–ESI–TQ–MS analysis under the supervision of Alexis V. Nathanail, who was also the corresponding author of this article.

ABBREVIATIONS

3Ac-DON	3-acetyl-deoxynivalenol
3Ac-T2	3-acetyl-T-2 toxin
15Ac-DON	15-acetyl-deoxynivalenol
A15	*Saccharomyces pastorianus* VTT A-63015
ADME	absorption, distribution, metabolism and excretion
ARfD	acute reference dose
bw	body weight
CAS	Chemical Abstracts Service
CM-82036	CM-82036-1TP-10Y-OST-10Y-OM-OFC (wheat variety)
CONTAM	Scientific panel on Contaminants in the Food Chain of EFSA
DNA	deoxyribonucleic acid
DOM-1	de-epoxy-deoxynivalenol
DON	deoxynivalenol
DON3Glc	deoxynivalenol-3-glucoside
EC	European Commission
EFSA	European Food Safety Authority
EIC	extracted ion chromatogram
ER	oestrogen receptor
ESI	electrospray ionisation
EU	European Union
FAO	Food and Agriculture Organization
FHB	*Fusarium* head blight
GC	gas chromatography
GIT	gastrointestinal tract
GSH	glutathione
GST	glutathione S-transferase
GT	glucosyltransferase
HCD	higher-energy collision dissociation
HILIC	hydrophilic interaction chromatography
HRMS	high resolution mass spectrometry
HT2	HT-2 toxin
HT2-3-Glc	HT-2 toxin-3-glucoside
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
MAPK	mitogen-activated protein kinase

MS	mass spectrometry
MS/MS	tandem mass spectrometry
m/z	mass-to-charge ratio
ML	maximum level
NIV	nivalenol
NIV3Glc	nivalenol-3-glucoside
NMR	nuclear magnetic resonance
PMTDI	provisional maximum tolerable daily intake
ppm	parts per million
QTOF	quadrupole time-of-flight
R^2	coefficient of determination
R_A	apparent recovery
RNA	ribonucleic acid
RSD	relative standard deviation
RSD_r	repeatability
RSD_R	precision
SIDA	stable isotope dilution assay
SIL	stable isotopic labelling
SPE	solid phase extraction
SRM	selected reaction monitoring
SSE	signal suppression/enhancement
TDI	tolerable daily intake
TIC	total ion chromatogram
TQ	triple quadrupole
T2	T-2 toxin
T2-(α-)Glc	T-2 toxin-(α-)glucoside
UDP-G	uridine diphosphate glucose
U(H)PLC	ultra (high) performance liquid chromatography
WHO	World Health Organization
ZEN	zearalenone
ZEN14Glc	zearalenone-14-glucoside
ZEN14Sulf	zearalenone-14-sulfate
ZEN16Glc	zearalenone-16-glucoside
α-ZEL	α-zearalenol
α-ZEL14Glc	α-zearalenol-14-glucoside
β-ZEL	β-zearalenol
β-ZEL14Glc	β-zearalenol-14-glucoside

"*If particulars are to have meaning, there must be universals.*"
Plato (c. 427 BC – c. 347 BC)

1 INTRODUCTION

The consumption of contaminated commodities with naturally abundant toxins and/or synthetic chemical toxicants may lead to disease outbreaks and chronic adverse health effects. Microorganisms are capable of producing a bewildering array of compounds that are toxic to humans and animals. One of the most important categories of microbial toxins, negatively impacting food and feed safety, is of fungal origin. Such toxic agents are defined as "mycotoxins" and are poisonous secondary metabolites produced by filamentous fungi (or moulds). Unlike man-made chemicals (e.g. synthetic pesticides), mycotoxins are naturally present and cannot be completely eliminated from the food chain.

Mycotoxins are low-molecular weight (below 1000 Da), chemically diverse, organic compounds that can induce a broad range of toxic effects in vertebrates, including acute toxicity, mutagenicity, carcinogenicity and immunotoxicity, as well as reproductive and specific target organ toxicities (nephrotoxicity, hepatotoxicity, neurotoxicity, etc.) (Steyn, 1995). The mycotoxin producers most relevant to food safety are *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp., but also members of the genera *Alternaria*, *Trichothecium*, *Cladosporium*, *Byssochlamys* and *Sclerotinia* are also of importance in food, because of potential mycotoxin production (Bullerman, 1979). Depending on a number of factors such as the temperature, relative humidity, water activity, geographic location, commodity and agricultural practices, favourable conditions for fungal invasion will most likely result in mycotoxin contamination of crops (Bhat et al., 2010). It has been hypothesised that many of these compounds may confer substantial selective advantages to fungi against other competing microorganisms (Magan and Aldred, 2007). Moreover, certain *Fusarium* mycotoxins such as the trichothecenes have been found to play an important role in plant pathogenesis (Desjardins and Hohn, 1997) and to act as virulence factors towards living plants by promoting infection (Harris et al., 1999; Proctor et al., 2002; Maier et al., 2006). Besides the ability of moulds to invade living plants in the field, they can also cause post-harvest infection of susceptible crops during storage and processing periods.

Survey studies have reported significantly high contamination estimates, with approximately 70% of food and feed cereal grain samples containing detectable amounts of at least one mycotoxin and 38% being co-contaminated with multiple mycotoxins (Schatzmayr and Streit, 2013). A very recent extensive metadata analysis, of more than 500,000 samples, revealed that 60–80% of cereals and nuts were found to contain detectable levels of mycotoxins (Eskola et al., 2019). Furthermore, according to the European Commission (EC), 5–10% of global crop production is lost annually because of severe mycotoxin contamination (EC, 2015). Due to the ubiquitous presence of mycotoxins in certain foods (e.g. cereals, nuts, vegetables and fruits) and feed, many

countries have established food legislation and regulatory limits that permit small mycotoxin concentrations assessed as low risk. In 2017, the Rapid Alert System for Food and Feed (RASFF) indicated that mycotoxins were the most frequently reported chemical hazard in the European Union (EU), a trend already appearing in earlier RASFF annual reports. More specifically, 29% of total border rejections in that year were because of mycotoxin contamination (predominantly aflatoxins) exceeding the EU's legislative limits (EC, 2018). These compounds are indisputably unavoidable contaminants in agricultural commodities that not only represent a direct risk to food and feed safety but can also have serious implications for food security and international trade (Krska et al., 2016).

However, exposure of humans and animals to mycotoxins is not the only risk factor associated with these compounds, as they may also exist in altered forms, the so-called "modified mycotoxins" (Rychlik et al., 2014), previously mainly referred to as "masked mycotoxins". Following its inception, the overarching term modified mycotoxins has been used to describe derivatives formed via phase I and II metabolic processes of plants, animals and fungi, as well as via the interaction of mycotoxins with food matrix components (Berthiller et al., 2013).

The issue of modified mycotoxins began to attract scientific attention after several mysterious cases of mycotoxicosis during the mid-1980s, in which toxicity symptoms in affected animals did not correlate with the low mycotoxin content detected in their feed. Around the same period, the *in planta* metabolic biotransformation of the *Fusarium* trichothecene mycotoxin deoxynivalenol (DON) to less toxic derivatives was for the first time hypothesised to occur in field corn inoculated with *F. graminearum* (Miller et al., 1983), and in naturally infected winter wheat (Scott et al., 1984). Deoxynivalenol-3-glucoside (DON3Glc) was later structurally characterised as the main phase II metabolite (Savard, 1991). Eventually, the two series of events were connected by associating clinical observations of the intoxicated animals with the existence of mycotoxin conjugates in animal feed, and the on-going research quest to study modified mycotoxins was thus initiated. Zearalenone (ZEN), another *Fusarium* mycotoxin, was the next piece to find its place in the modified mycotoxin puzzle when maize cell cultures were found capable of transforming ZEN to zearalenone-14-glucoside (ZEN14Glc) and other metabolites (Engelhardt et al., 1988).

These findings were first put into a toxicological perspective by Gareis et al., (1990), who described the complete *in vivo* release of the aglycone in pigs fed with ZEN14Glc-contaminated feed. Although research on modified mycotoxins since that time has grown considerably, the toxicological relevance of these compounds to humans and animals still remains largely unknown. Certain modified forms of mycotoxins, such as α-zearalenol (α-ZEL), a major phase I metabolite of ZEN in animals and plants, may be more hazardous than their precursors, whilst others are less hazardous, such as β-zearalenol (β-ZEL) (Zinedine et al., 2007). On the other hand, phase

II metabolic reactions resulting in conjugation with polar metabolites such as carbohydrates or sulfate are generally viewed as detoxification processes. The limited existing toxicity studies, performed almost exclusively with DON3Glc, are principally in agreement that conjugated modified mycotoxins possess a significantly reduced direct toxicity compared to precursor forms (Gratz, 2017). Regardless of the suspected low intrinsic toxic potential of these mycotoxin derivatives, they may be manifesting toxicological implications via other mechanisms, for instance by releasing the toxic precursor during digestion or food manufacturing processes.

Currently, there is no regulatory framework covering modified mycotoxins. In 2011, the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) decided to include the acetylated derivatives of DON, 3-acetyl-DON (3Ac-DON) and 15-acetyl-DON (15Ac-DON), in its existing provisional maximum tolerable daily intake (PMTDI) of 1 μg/kg body weight (bw) (JECFA, 2011). In the EU, the European Food Safety Authority (EFSA) has lately been actively engaged in assessing the risks associated with modified mycotoxins and establishing health-based guidance values for groups of individual *Fusarium* mycotoxins together with their modified forms. It is worth mentioning, that both scientific bodies have explicitly emphasised the urgent need for further work on the identification of as yet uncharacterised modified mycotoxins, the generation of more occurrence data in food and feed for known modified mycotoxins and investigations into their toxicokinetics and toxicity.

The tremendous progress that has occurred during the past couple of decades in the area of analytical chemistry has provided scientists with an arsenal of modern high-throughput instruments that can be utilised to address numerous analytical challenges related to most contaminants relevant to food and feed safety. Undoubtedly, research concerning mycotoxins and presently modified mycotoxins has taken advantage of these developments (Krska et al., 2017). Hyphenated analytical techniques and more specifically liquid chromatography–mass spectrometry (LC–MS)-based methods are now routinely used not only for the concurrent targeted determination of multiple mycotoxins in various sample matrices, but also in metabolomics studies for the identification of novel mycotoxins and metabolites thereof (Malachová et al., 2017). In recent years, the use of high resolution mass spectrometry (HRMS) analysers has been pivotal in mycotoxin research, allowing the accurate, non-targeted identification of modified mycotoxins, even retrospectively (Righetti et al., 2016). In conjunction with advancements in the development of analytical instrumentation, bioinformatics software packages that can rapidly process large quantities of raw LC–HRMS data have emerged. These powerful tools are becoming indispensable in metabolic profiling, yielding insights into the interactions of complex biological systems such as plants and microorganisms.

Nevertheless, safety assessment on the relevance of modified mycotoxins to human and animal health constitutes a perplexing endeavour for a number of reasons. First, for each native mycotoxin present in food or feed, potentially dozens of modifications may co-exist. These mixtures of various mycotoxins and their modified forms can cause unpredictable combined toxic effects upon exposure (Nathanail et al., 2016). Secondly, an unequivocal bottleneck in modified mycotoxin research is the absence of reference standards for all but a handful of these compounds, which are necessary to allow further investigations and quantification in commodities (Broekaert et al., 2015). Finally, only limited toxicokinetic and toxicity data are available to allow a better understanding of the toxicological behaviour of the few sufficiently characterised modified mycotoxins, which is likely to differ from that of their precursors (De Boevre et al., 2015). Hence, there is a wealth of information still to be generated in this area.

The original contribution of this Ph.D. dissertation was in shedding some light on a number of the manifold unexplored areas related to modified mycotoxins. In particular, the main aims of the thesis were to present an extensive literature overview of key aspects related to modified *Fusarium* mycotoxins and study their formation, natural occurrence and metabolic fate. To achieve the research goals, reliable, sensitive and accurate analytical LC–MS-based techniques were developed and validated, which were capable of simultaneously determining several modified mycotoxins together with their precursors. Such state-of-the-art methodology was used to generate information on the natural occurrence of modified mycotoxins in Finnish cereal grains for the first time. Another highlight of this work was the identification of novel modified forms following exposure of barley and wheat to the *Fusarium* trichothecene mycotoxins HT-2 toxin (HT2) and T-2 toxin (T2), and to eventually provide new insights into plant–mycotoxin interactions and the detoxification pathways involved. Finally, investigations were conducted to assess the impact of high doses of the trichothecenes DON, HT2 and T2, as well as DON3Glc on brewing yeast and to examine the potential formation and/or release of conjugated moieties during fermentation.

2 LITERATURE REVIEW

2.1 *Fusarium* fungi and mycotoxins

Fungal inoculum can enter the food chain either while crops are growing in the field, via the colonisation of living plants by parasitic fungi, or by spoilage fungi after crops have been harvested. *Fusarium*, first described and defined by Link (1809) as *Fusisporium*, is a form genus of ascomycete fungi. Many *Fusarium* species are notorious plant pathogens and toxin producers, affecting agriculture and horticulture on a global scale. These moulds can also act as direct pathogens to humans and some domesticated animals, with members of *Fusarium oxysporum* and *F. solani* commonly being associated with keratitis (Bullock and Khamis, 2010) and skin and nail infections in immunocompromised individuals (Gupta et al., 2000).

In Europe, the main host plants of fusaria are cereal grains and potatoes, on which they may cause seed, head and leaf blight, root, crown, head and cob rot, as well as storage rot in tubers and kernels (Nirenberg, 1989). Plant diseases such as *Fusarium* head blight (FHB) that are associated with *Fusarium* spp. can be devastating for small grain cereals (wheat, barley, oats, rye and triticale). Numerous publications have underlined the massive economic impact that *Fusarium* infections can have in cereal production and food security, deteriorating quality and reducing grain yields (Parry et al., 1995; Bottalico and Perrone, 2002; Chakraborty and Newton, 2011). Fusaria can also produce a variety of phytotoxic metabolites upon infestation of plant tissue that act as virulence or pathogenicity factors, interfering with plant defence responses and facilitating fungal colonisation on host plants. Some of these compounds may also be detrimental to human and animal health and thus classified as mycotoxins.

As a consequence, contamination of food and feed with mycotoxins produced by *Fusarium* species, also known as fusariotoxins, may cause intoxications, especially in farm animals. More specifically, cereal grains used for human consumption and animal feed are subject to contamination with trichothecenes, ZEN and fumonisins, all of which are regarded as major classes of *Fusarium* mycotoxins (D'mello et al., 1999). All three major classes were discovered during the 30-year golden period of *Fusarium* mycotoxicology between 1961 and 1991 (Desjardins, 2006). By the end of this period, toxicologists had already unravelled their mechanisms of toxicity: trichothecenes, inhibition of protein synthesis; ZEN, binding to the oestrogen receptor (ER); and fumonisins, inhibition of sphingolipid biosynthesis. Other *Fusarium* mycotoxins, often referred to as "emerging" mycotoxins, such as fusaproliferin, beauvericin and moniliformin (reviewed in Jestoi, 2008), were also characterised at that time. It should

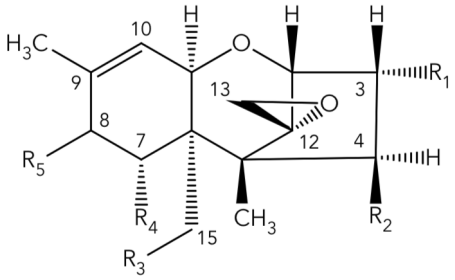
be noted that the present work principally focused on the trichothecene mycotoxins HT2, T2, DON and nivalenol (NIV), as well as ZEN and many of their known derivatives.

2.1.1 Trichothecenes

The trichothecenes are large group of more than 200 chemically related mycotoxins (Grovey, 2007), produced by various species of *Fusarium*, *Myrothecium*, *Spicellum*, *Stachybotrys*, *Cephalosporium*, *Trichoderma* and *Trichothecium* (Cole et al., 2003). *Fusarium* spp. predominantly associated with trichothecene production in Europe are *F. graminearum*, *F. culmorum*, *F. sporotrichioides* and *F. poae*, among others (Bottalico, 1998). The trichothecene biosynthetic pathway requires the expression of a transporter protein and a network of regulatory genes to form the tricyclic sesquiterpene backbone with an olefinic bond between carbons 9 (C-9) and 10, as well as an epoxide ring at C-12,13 (Desjardins and Proctor, 2007). Trichothecenes are non-volatile, low-molecular weight, heat-stable compounds (Hazel and Patel, 2004) and are divided into four groups (types A–D), according to their chemical properties and producer fungi (Ueno et al., 1973).

The main type A trichothecenes are HT2 (Chemical Abstracts Service; CAS No. 26934-87-2; $C_{22}H_{32}O_8$) and T2 (CAS No. 21259-20-1; $C_{24}H_{34}O_9$) and do not contain a carbonyl group at the C-8 position, in contrast to type B, represented primarily by DON (CAS No. 51481-10-8; $C_{15}H_{20}O_6$) and NIV (CAS No. 23282-20-4; $C_{15}H_{20}O_7$), which do possess a carbonyl function at C-8 (Figure 1). Type C trichothecenes (e.g. crotocin and baccharin) are characterised by a second epoxide function at C-7,8 or C-9,10. Lastly, trichothecenes of type D (e.g. satratoxin and roridin) contain a macrocyclic ring between C-4 and C-15 (Ueno, 1980).

Of all *Fusarium* mycotoxins known to date, trichothecenes have been most strongly linked with mycotoxicoses of humans and animals in Europe. The acute toxicity of trichothecenes varies somewhat, but type A trichothecenes generally tend to be more acutely toxic than type B, with a decreasing order of toxicity from isovaleryl > hydrogen > hydroxyl substitutions at the C-8 position (Rotter, 1996). Due to the low molecular weight and amphipathic nature of trichothecenes, integumentary and gastrointestinal absorption occur rapidly, resulting in systemic exposure and subsequent toxic effects especially on proliferating tissues (McCormick et al., 2011). From a toxicological perspective, trichothecenes are recognised as having multiple effects on eukaryote cells, including protein, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis (Thompson and Wannemacher Jr, 1986; Shifrin and Anderson, 1999) and the disruption of mitochondrial metabolism (Minervini et al., 2004), as well as effects on cell division and membrane function (Bunner and Morris, 1988; Rocha et al., 2005). The underlying molecular mechanisms of action are now mostly well documented.



Trichothecene		Molecular weight (g/mol)	R_1	R_2	R_3	R_4	R_5
Type A	HT2	424.48	–OH	–OH	$–OCOCH_3$	–H	$–OCOCH_2CH(CH_3)_2$
	T2	466.52	–OH	$–OCOCH_3$	$–OCOCH_3$	–H	$–OCOCH_2CH(CH_3)_2$
Type B	DON	296.32	–OH	–H	–OH	–OH	=O
	NIV	312.32	–OH	–OH	–OH	–OH	=O

Figure 1. Chemical structures of HT2, T2, DON and NIV with carbon numbering of the tetracyclic trichothecene skeleton.

A number of *in vitro* assays have concluded that although trichothecenes have a broad range of biological effects, their major mode of action is the inhibition of protein synthesis in a large number of mammalian cell lines tested (reviewed by Gutleb et al., 2002). At the cellular level, the common chemical scaffold of trichothecene inhibitors moderates their binding to the A-site of the peptidyl transferase centre of the 60S subunit of eukaryotic ribosomes, interfering with protein translation (de Loubresse et al., 2014). A ribotoxic stress response induced by exposure to trichothecenes, such as DON, is then typically induced, eliciting the activation of mitogen-activated protein kinases (MAPKs) and up-regulation of pro-inflammatory cytokines and chemokines (Pestka, 2010; Cano et al., 2013; Zhang et al., 2016). These complex effects may evoke immunostimulation or immunosuppression, depending on the dosage, duration and frequency of exposure, or trigger cell signalling cascades ultimately mediating apoptosis (Pestka et al., 2004). In addition, DON and T2 have been found to inhibit protein synthesis in ribosomes from wheat and maize (Casale and Hart, 1988). These toxins are known to act as virulence factors in disease development by inducing reactive oxygen species, which in turn stimulate apoptosis and assist necrotrophic fungal growth (Nishiuchi et al., 2006; Desmond et al., 2008). There is sufficient scientific evidence suggesting the phytotoxicity of trichothecenes produced by *Fusarium* spp., causing necrosis, chlorosis and mortality and enabling them to mediate a variety of plant diseases such as stalk, root and leaf rot, as well as wilts (Ismaiel and Papenbrock, 2015).

Among all the trichothecenes identified to date, only a few type A and B toxins are of relevance to food and feed safety because of their prevalence in crops. T2 is the most acutely toxic trichothecene to humans and animals and can trigger anorectic effects and emetic events upon single exposure, as can HT2 (EFSA, 2011a). *In vivo* tests have shown that T2 causes haemato- and myelotoxicity and is associated with an

increased infection rate, DNA damage and the induction of apoptosis, and has been found responsible for outbreaks of alimentary toxic aleukia (ATA) (Li et al., 2011). The characteristic symptoms of ATA are fever, haemorrhage, necrosis, leukopenia and exhaustion of bone marrow, resulting in up to 60% mortality for this endemic foodborne disease (Ueno, 1980). Probably one of the most hazardous food-associated mycotoxins, partly also due to its high prevalence, is DON, which is able to cross biological membranes and provoke intoxications characterised by vomiting, anorexia, abdominal pain, diarrhoea and headaches (Maresca, 2013). Fungal metabolites of DON such as 3Ac-DON and 15Ac-DON can also be present in food- and feedstuffs. These modified forms of DON may be reverted to the parent toxin by gut microbiota during digestion or may exert intrinsic toxicity; for certain endpoints, 15Ac-DON can be more toxic than DON (Pinton et al., 2012). Comparably to the other trichothecenes, NIV toxicity is manifested by hepatotoxicity, myelotoxicity and immunotoxicity, as well as developmental toxicity (EFSA, 2013). A testament to their toxicity is epidemiological evidence and intelligence assessments supporting the contention that trichothecenes have been used as biological warfare agents in military operations in Southeast Asia and Afghanistan (Wannemacher Jr et al., 1997).

Apart from the trichothecene mycotoxins being discussed in this thesis, other trichothecenes are also toxicologically important contaminants in cereals (e.g. diacetoxyscirpenol) and in indoor air (e.g. satratoxin H). However, currently unidentified mycotoxins may potentially be present in crops or start appearing as a consequence of selection pressure from resistant plants on fungal populations, with serious implications for food safety. Recently, several newly identified strains of *F. graminearum* were isolated in North American wheat that were capable of producing novel type A trichothecenes (Varga et al., 2015). These mycotoxins were labelled as NX-2 and NX-3.

The extent to which the co-occurrence of several *Fusarium* mycotoxins and their modified forms, in cereal grains and animal feed, might enhance toxic effects has not hitherto been possible to quantify. Furthermore, information on chronic effects resulting from exposure to single or mixtures of mycotoxins is also very limited.

2.1.2 Zearalenone

ZEN (CAS No. 17924-92-4; $C_{18}H_{22}O_5$), is a low-molecular-weight (318 g/mol), non-steroidal oestrogenic mycotoxin. ZEN is heat stable, with its degradation occurring at temperatures higher than 150 °C or under alkaline conditions (Ryu et al., 2003). It is biosynthesised via a polyketide pathway by a variety of *Fusarium* spp., including *F. graminearum*, *F. culmorum*, *F. cerealis* and *F. equiseti*. Chemically, ZEN is a resorcyclic acid lactone (Figure 2), containing an ester group (C-1), one ketonic group (C-7), one olefinic group (C-11,12) and two phenolic hydroxyls (C-14 and C-16) (Urry et al., 1966).

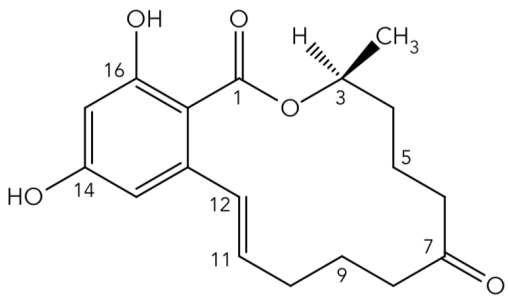


Figure 2. Chemical structure of ZEN with carbon numbering.

ZEN is rapidly and extensively absorbed in the mammalian gastrointestinal tract (GIT). During metabolism of ZEN, the keto group at C-7 is reduced, a reaction mediated by 3α- and 3β-hydroxysteroid dehydrogenases, forming two stereoisomeric metabolites: α-ZEL and β-ZEL (Minervini et al., 2008). These metabolites can also be formed by fungi and plants (Bottalico et al., 1985; Berthiller et al., 2006). ZENs are not acutely toxic and do not cause fatal mycotoxicoses in humans or animals, having oral median lethal doses (LD_{50}) of > 2000–20,000 mg/kg bw in different animal models (JECFA, 2000). However, they have been associated with oestrogenic effects, manifesting as hyperoestrogenism. ZEN and some of its phase I metabolites constitute an important class of endocrine-disrupting chemicals that have been shown, in several *in vitro* and *in vivo* test systems, to competitively bind to ER-α and ER-β, inducing oestrogenic-like effects by activating gene transcription via oestrogen-responsive elements (Zinedine et al., 2007). The highest binding affinity to oestrogen receptors and subsequent oestrogenic effects has been reported to be α-ZEL > ZEN > β-ZEL (Frizzell et al., 2011; Fitzpatrick et al., 1989). Swine is one of the most sensitive species to ZENs, where direct ingestion of contaminated feed causes enlargement of the mammary glands and organs of the genital tract, atrophy of ovaries, infertility and other reproductive effects in females, as well as enlargement of the mammary glands and atrophy of the testes in males (Desjardins, 2006). ZEN has also been found to be clastogenic in a variety of cell culture systems and capable of inducing micronuclei in bone marrow cells and chromosomal aberrations *in vivo* (EFSA, 2011b). The formation of ZEN/α-ZEL catechol metabolites that elicit oxidative DNA damage has been associated with the observed genotoxic effects of the mycotoxin (Fleck et al., 2012).

Regarding plant effects, ZEN appears to be generally less phytotoxic compared to certain trichothecenes (Ismaiel and Papenbrock, 2015). Nevertheless, ZEN can affect plant growth and development, as well as the functioning of the photosynthetic apparatus (Kościelniak et al., 2009). Furthermore, ZEN and its α- and β-ZEL derivatives caused the leakage of electrolytes and root cell death in two maize (*Zea mays* L.) cultivars (Repka et al., 2014). In another study, when mature maize embryos were exposed to a

mixture of ZEN and DON, additive and possibly synergistic inhibition of root and shoot elongation was observed compared to DON alone (McLean, 1995).

2.2 Modified *Fusarium* mycotoxins

2.2.1 Nomenclature

Mycotoxins alone do not epitomise the only health hazard for humans and animals as derivatives of them, originating from a number of different sources, can still be present in food and feed. Mycotoxins chemically altered via metabolic processes or, abiotically, via interactions with food matrix components may co-exist together with native forms at significant concentrations (Berthiller et al., 2013; Freire and Sant'Ana, 2017). These mycotoxin derivatives may exhibit a significantly different chemical and toxicological behaviour compared to the parent molecules and have been in the spotlight of mycotoxin research during the past decade or so.

Following the formulation of a working hypothesis by Miller et al. (1983) regarding the formation of "unknown metabolites" by plants exposed to mycotoxins, the next key scientific discovery in the area was accomplished with the introduction of the term "masked mycotoxins" (Gareis et al. 1990). This term was originally intended to emphasise the presence of certain mycotoxin metabolites and the role they could play in cases of animal mycotoxicoses, where there was no correlation between toxicity symptoms in affected animals and the mycotoxin content detected in their feed. It also highlighted the fact that such mycotoxin derivatives were, at the time, undetectable by routine analytical techniques. However, as analytical methodology has rapidly evolved and simultaneously altered mycotoxins are constantly being characterised, previously unknown/masked mycotoxins are becoming unmasked/known.

Since the introduction of the term "masked mycotoxins", it has been ambiguously used in the literature to describe all types of mycotoxin structural alterations. Terminology including "hidden", "conjugated" and "bound" mycotoxins has also been used for similar and often overlapping purposes (Berthiller et al., 2016). In order to address the issue of inconsistent nomenclature, Rychlik et al. (2014) published a systematic definition consisting of four hierarchic levels. According to that new definition, distinctions were made at the highest level between "free" (e.g. DON), "matrix-associated" (e.g. DON-oligosaccharides) and "modified" mycotoxins. In the next hierarchical levels, modified mycotoxins were further subdivided into two categories: those biologically conjugated by plants (e.g. DON3Glc; Figure 3), animals (e.g. DON-3-glucuronide) or fungi (e.g. zearalenone-14-sulfate, ZEN14Sulf) and those chemically modified thermally (e.g. norDON), non-thermally (e.g. DON-sulfonate) or

differently (e.g. de-epoxy-deoxynivalenol, DOM-1). As a side note, for mycotoxins biologically modified by plants, the term "masked" is still acceptable as a synonym. In an attempt to harmonise future references to altered mycotoxins, EFSA adopted the definition of "modified" mycotoxins and the above categorisation system (EFSA, 2014). Furthermore, EFSA recommended in the same opinion the utilisation of an unequivocal system of abbreviations and chemical structure numbering for both parent and modified mycotoxins, to resolve complications arising from inconsistent naming in the scientific literature.

In this thesis, the term "modified mycotoxins" is used to refer to compounds resulting from biologically-induced alterations in mycotoxin structures regardless of their origin, whilst unaltered mycotoxins are referred to interchangeably as "native", "precursor" or "parent". Additionally, both the abbreviation and numbering system proposed by EFSA in 2014 have been implemented.

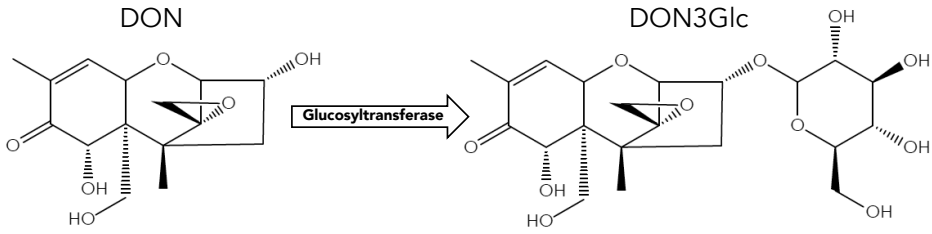


Figure 3. *In planta* formation of modified mycotoxins, e.g. by conversion of DON into DON3Glc by glucosyltransferases.

2.2.2 Formation by plants and fungi

Mycotoxins can be structurally modified by plants, microorganisms, mammals and food manufacturing processes. Here, modified derivatives of mycotoxins arising from interactions with plants and moulds are mainly discussed.

Plants are equipped with versatile detoxification systems to counteract the deleterious effects of natural and synthetic chemicals or xenobiotics. Xenobiotics that contain electrophilic sites are the ones that are particularly hazardous to all organisms. These molecules can exert toxic effects by covalent binding to nucleophilic sites (e.g. DNA and proteins). Many xenobiotics are lipophilic, readily absorbed and can accumulate to toxic levels within plant cells, unless effective detoxification takes place. The metabolic cascade employed by plants for protection from toxic compounds can be divided into three stages: i) phase I activation reactions (usually oxidation and hydrolysis), ii) phase II conjugation reactions with endogenous substances (e.g. glucose, glutathione (GSH), amino acids, etc.) and iii) phase III compartmentation processes (Coleman et al., 1997).

During phase I metabolism, reactive or polar groups are introduced to the xenobiotic by enzymatic action. However, if a xenobiotic already has a reactive site suitable for conjugation, this stage may then be omitted. The cytochrome P450 monooxygenases (P450s) play an important role in the phase I metabolism (oxidation) of xenobiotics and exist as integral membrane proteins in the endoplasmic reticulum of plant cells (Schuler, 1996). Esterases and amidases catalyse hydrolysis reactions. Because phase I reactions are in principal activating, the resulting metabolites may not always be less phytotoxic than the precursor molecules.

Phase II biotransformation reactions involve the covalent linkage of xenobiotics to various endogenous molecules, giving rise to end-products with increased hydrophilicity and molecular weight, as well as with altered chemical and toxicological properties. Glucosylation (i.e. conjugation to a glucose moiety) is one of the primary mechanisms that plants utilise to maintain metabolic homeostasis and to detoxify xenobiotics (Jones and Vogt, 2001). Lipophilic small-molecule acceptors are glucosylated at –OH, –COOH, $–NH_2$, –SH and C–C groups by glucosyltransferase (GT) enzymes, particularly of family 1 (Bowles et al., 2006); the activated sugar donor of plant GTs for xenobiotics is primarily uridine diphosphate glucose (UDP-G). Generally, glucosylation transforms toxins into stable non-reactive storage forms with increased water solubility and reduced toxicity for the plant. Historically, ZEN was the first mycotoxin for which conjugation to the β-D-glucopyranoside was reported and characterised as ZEN14Glc in cereal cell cultures by Engelhardt et al. (1988). The other mono-glucosylated isomer of ZEN, zearalenone-16-glucoside (ZEN16Glc), was also much later described in the literature (Kovalsky Paris et al., 2014). *In planta* glucosylation of *Fusarium* mycotoxins are commonly β-linked glucose conjugates (e.g. DON3Glc, nivalenol-3-glucoside (NIV3Glc) and ZEN14/16Glc), whereas both α- and β-glucoside conjugates have been reported for T2 (Gratz, 2017).

Conjugation of xenobiotics with GSH, the tripeptide γ-glutamyl-cysteinyl-glycine, is another common detoxification pathway in plants, frequently targeting electrophilic, hydrophobic and toxic compounds. GSH is abundant in plants (> 1 mM in the cytoplasm), and the conjugation of endogenous phytochemicals and exogenous toxins with it is either spontaneous or mediated by glutathione S-transferases (GSTs) (Dixon et al., 1998). These conjugations are generally substitution-based reactions, and less often addition reactions, which have been found to play an important role in the selective tolerance of plants towards herbicides (Edwards et al., 2000). Other chemical modifications may occur in plants prior to compartmentation, involving the addition of moieties such as of malonic acid, sulfate, amino acids and more.

Finally, for phase III, the major pathways known thus far to exist in plants are for the metabolites to be exported into cell vacuoles or into the extracellular space, or to be deposited into lignin and other cell wall components (Sandermann Jr, 1992). The addition of hydrophilic moieties onto lipophilic toxins prevents passive diffusion across

lipid bilayers between cellular compartments, thus making carrier systems necessary for the transport of metabolites, which are most likely to vary depending on the type of conjugated moiety (Berthiller et al., 2013). As a consequence of compartmentation, the conjugated toxic substances are prevented from interacting with cellular components and exerting their toxicity to the plant.

Hence, modified mycotoxins can be formed by plant defence mechanisms as a physiological response to fungal infection. The majority of characterised modified mycotoxins are conjugated metabolites of parent fungal metabolites (mycotoxins) with polar compounds (EFSA, 2014). Glucosylation, sulfation, acetylation and GSH conjugation are the most frequently reported pathways involved in the formation of modified mycotoxins by plants and fungi (Berthiller et al., 2009). These pathways are not unique to mycotoxins, and as previously described, they are often succeeded by compartmentation processes in plants. Figure 4 illustrates two examples of *in planta* enzyme-catalysed detoxification reactions (GSH conjugation and glucosylation) of mycotoxins.

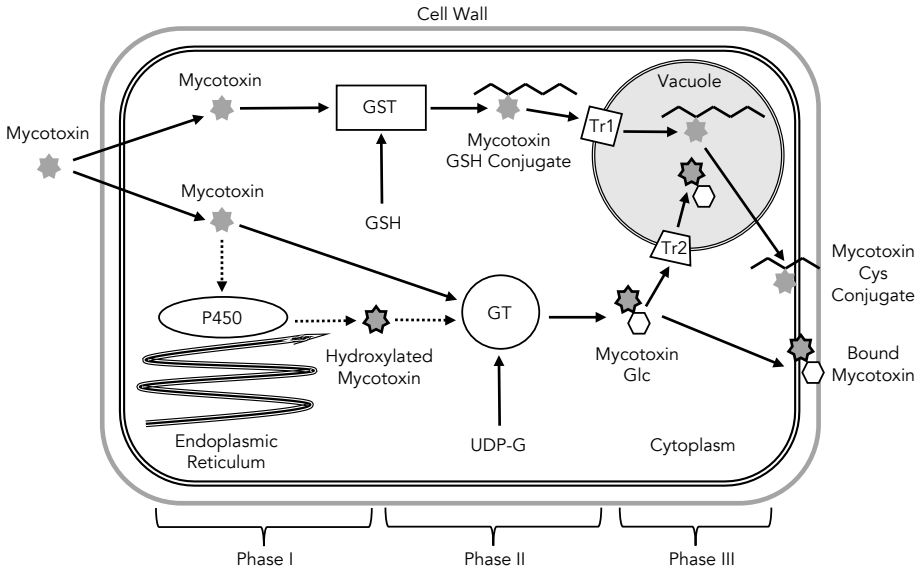


Figure 4. The three-stage enzyme-catalysed detoxification process of mycotoxins in plants. The broken arrow indicates optional metabolic steps that may not be necessary for conjugation reactions to take place. Adapted from Coleman et al., 1997.
Abbreviations: P450, cytochrome P450 monooxygenases; GST, glutathione S-transferase; GSH, glutathione; GT, glucosyltransferase; UDP-G, uridine diphosphate glucose; Glc, glucoside; Tr1, glutathione-conjugate transporter; Tr2, glucose-conjugate transporter; Cys, cysteine.

Fungi can also contribute to the modified mycotoxin load of commodities, since they are capable of either directly excreting modified mycotoxins or metabolising existing mycotoxins (Broekaert et al., 2015). The acetylated DON derivatives, 3Ac-DON

and 15Ac-DON, can be directly excreted by fungi; both metabolites are biosynthetic precursors of DON and arise from a common precursor (3,15-diacetyl DON), and are also used as genetic markers to differentiate between 3Ac-DON and 15Ac-DON chemotypes in *Fusarium* (Alexander et al., 2011). Moreover, moulds can metabolise existing mycotoxins produced toxigenic species. Namely, ZEN14Sulf was first isolated from a rice culture of *F. graminearum* and reported by Plasencia and Mirocha (1991). Fungi of the genera *Rhizopus* and *Aspergillus* are also able to biotransform ZEN to ZEN14Sulf (El-Sharkaway et al., 1991; Brodehl et al., 2014). In addition, fungal glucosylations of the *Fusarium* mycotoxins ZEN, HT2, T2 and DON to ZEN14Glc, HT-2 toxin-3-glucoside (HT2-3-Glc), T-2 toxin-glucoside (T2-Glc) and DON3Glc, respectively, have been described in the literature (Kamimura, 1986; Busman et al., 2011; Tian et al., 2016).

2.2.3 Transformations via food processing and brewing

A large proportion of the harvested cereal grains are further processed into various products, including flour, bread, pasta, bread and beer. Food processing can impact on mycotoxins present in raw materials by physical removal, adsorption to solid surfaces, as well as chemical and enzymatic transformations. Furthermore, the transfer of heat during food manufacturing processes can cause either release of the precursor mycotoxins or reactions with food macromolecules such as sugars, proteins and lipids (Suman and Generotti, 2016). Large variations in the levels and chemical structures of mycotoxins and modified forms have been documented for cleaning, milling, brewing, baking, (alkaline) cooking, fermentation, roasting and extrusion (reviewed in Karlovsky et al., 2016). Hence, knowledge of the chemical fate of mycotoxins under various types of food processing operations is of utmost importance in ensuring the safety of foodstuffs. As regards food processing, this Ph.D. dissertation focused on investigating the fate of mycotoxins during beer fermentation and their impact on brewing yeast.

Beer is the most consumed alcoholic beverage in the world and can be subjected to mycotoxin contamination, predominantly due to infected raw materials. Beer production involves a variety of procedures that might impact on the initial level of mycotoxins in a positive or negative way (Pascari et al., 2018). The two main processes in beer making are malting and brewing. During malting, which consists of three stages (steeping, germination and kilning), raw barley or another grain is steeped in water to obtain the correct moisture content and allowed to germinate under precise conditions to produce malt. The malt is then soaked into brewing liquor (mashing) and the temperature is raised to produce the liquid extract (wort), which is then separated from the insoluble material (spent grains). The other important step in brewing is fermentation, upon which the sugary wort is converted into beer by producing alcohol,

carbon dioxide, plus some amount of other flavouring metabolic by-products (Willaert, 2007). Beer fermentation is a process initiated by yeast primarily of the *Saccharomyces* genus: ale or top fermentation using *S. cerevisiae* and lager or bottom fermentation using *S. pastorianus* (Pascari et al., 2018). A number of studies have investigated the transfer of mycotoxins, particularly trichothecenes and ZEN, from the starting cereal commodities (barley and malt) into the final beer-making chain. Generally, DON is a mycotoxin frequently found in different beers together with DON3Glc, whereas NIV, HT2, T2, ZEN and fumonisins have rather rarely been documented and/or at lower concentrations (Varga et al., 2013; Peters et al., 2017).

Lancova and co-workers (2008) reported that the levels of *Fusarium* mycotoxins tended to significantly decrease during the steeping step. This is due to the treatment of grains with quite a high amount of water, which removes a proportion of water-soluble mycotoxins from the outer matrix surface (Schwarz et al., 1995). During the germination process, mycotoxins may undergo extensive structural transformations. For instance, the DON content was reduced by 50% with an almost equal amount of DON3Glc formation, because of the high enzymatic activity in germinating grains (Maul et al., 2012). Nevertheless, *de novo Fusarium* micromycete growth accompanied by mycotoxin production may occur in this stage of brewing, resulting in significantly increased mycotoxin levels (Lancova et al., 2008). According to the same paper, kilning appears to contribute minimally to mycotoxin levels. On the contrary, mashing is a very crucial step influencing the mycotoxin concentration dynamics in brewing. Under certain conditions, technological parameters such as temperature, the ratio of water to raw material and enzymatic activity can dramatically increase mycotoxin concentrations (levels as high as 600% of DON content in malt have been reported) (Niessen et al., 1993). Nevertheless, the key factor concerning the mycotoxin content in the final beer product is the starting malt contamination (Kostelanska et al., 2011).

Finally, yeast fermentation may also be affected by the fungal metabolites in the wort. Decreases in the rate of fermentation by *S. cerevisiae* have been recorded in fermentation broths containing 10–50 μg/g T2, 5–50 μg/g ZEN and 50 μg/g DON (Wolf-Hall, 2007). However, there was no effect at 20 μg/g DON. NIV at levels below 50 μg/g was also found not to have an effect on ale or lager yeast strains (Boeira et al., 1999), and only at very high concentrations was a synergistic interaction between DON and ZEN observed (Boeira et al., 2000). Yeast fermentation can also affect mycotoxin concentrations by adsorption to the yeast cell wall and metabolism (Karlovsky et al., 2016). To sum up, beer is undoubtedly affected by mycotoxin contamination and beer production processes can influence the mycotoxin load and accumulated forms to a great extent. Several parameters have been found to cause an inhibitory effect on mycotoxin levels, namely the starting raw material contamination, steeping, mashing and fermentation.

2.3 Toxicological ramifications of modified *Fusarium* mycotoxins

Modified mycotoxins can substantially differ in toxicokinetic and toxicodynamic parameters compared to their precursors, due to often marked structural differences and the resulting physico-chemical properties (polarity, solubility, etc.). Humans are principally exposed to mycotoxins and derivatives thereof via contaminated foodstuffs, whereas the lungs and skin might only represent a possible route in certain occupational cases (e.g. farmers and bakers); animals are exposed via feed. Following the consumption of contaminated commodities, modified mycotoxins may either become metabolically formed within an organism or re-activated in the GIT, and potentially exert adverse effects on target organ(s)/system(s). Nevertheless, evaluation of any additional health burden arising from modified mycotoxins is hindered by the poor availability of exposure and toxicity data, even though the literature is rich in reports concerning the toxicological profiles of unmodified mycotoxins. Figure 5 outlines the theoretical fate of modified mycotoxins in monogastric species based on current knowledge.

Pertaining to the toxicological significance of modified mycotoxins, current research has focused on i) understanding their chemical/metabolic fate during food processing or digestion and ii) investigating their intrinsic toxicity. Due to the complexity and high cost of animal studies, in addition to the limited availability of pure modified mycotoxins, the conducting of comprehensive *in vivo* investigations is not favoured. Currently, only a few *in vivo* studies on modified mycotoxins are available, which do provide, however, very useful information concerning the metabolic fate and systemic toxicity of these compounds. On the other hand, non-animal test methods (e.g. *in silico* and *in vitro*; reviewed in Nathanail et al., 2016) are powerful assays that can effectively assist in overcoming several of the drawbacks associated with *in vivo* experiments, and grant researchers the necessary flexibility and reliability to keep pace with the constantly rising number of identified (modified) mycotoxins. To date, the bulk of available toxicity data on plant and fungal modified *Fusarium* mycotoxins have essentially targeted the 3-glucoside of DON and some acetylated DON derivatives, accompanied by a number of ZEN-modified forms (Broekaert et al., 2015).

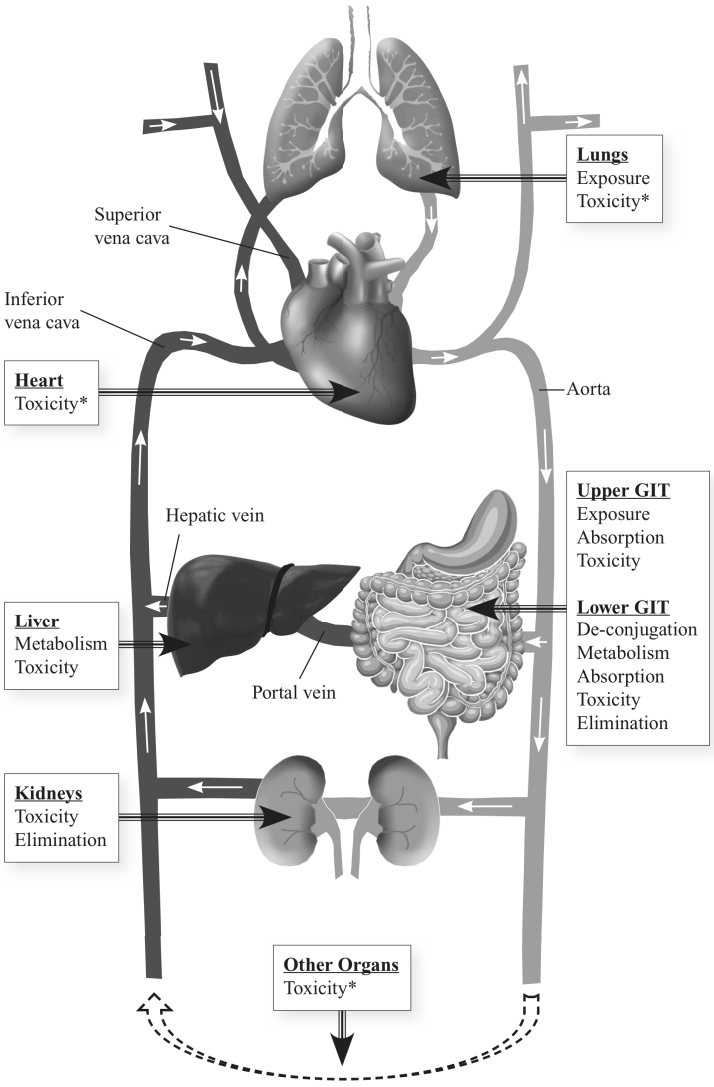


Figure 5. Theoretical fate of modified mycotoxins in monogastric species.
**Unconfirmed*.
Original source: Nathanail et al., 2016; © The Royal Society of Chemistry 2016.

For DON, an *in silico* analysis was recently performed to investigate the impact of glucosylation on the manifestation of its toxicity (Pierron et al., 2016). It was demonstrated that DON3Glc is sterically unable to bind to the A-site of the ribosomal peptidyl transferase centre, in contrast to its parent. In a follow-up experiment by the same authors, the cell viability and barrier function of Caco-2 cells were unaffected by exposure to DON3Glc, and it did not activate JNK or P38 MAPKs, the activation of which mediates apoptosis and can cause up-regulation of pro-inflammatory cytokines and chemokines (Pestka, 2010). Similar findings from other *in vitro* studies verified the absence of adverse

toxic effects of DON3Glc, reporting no impairment of Caco-2 cell viability (up to 16.8 μM DON3Glc) (Alizadeh et al., 2016) and no cytotoxicity in IPEC-J2 porcine intestinal cells (Broekaert et al., 2016). Microarray analysis of 30,000 genes revealed that the treatment of intestinal explants with 10 μM DON3Glc did not change the expression or cause any histomorphological alterations. By contrast, 10 μM DON caused morphological lesions and up-regulation of genes of pro-inflammatory cytokines (Pierron et al., 2016). It thus appears that the size increase of DON3Glc in addition to the glucosylated hydroxyl group at C-3, essential for binding to the ribosome, renders this modified mycotoxin significantly less toxicologically active. DON3Glc has also been shown to have a strongly reduced ability to inhibit protein synthesis in wheat ribosomes compared to DON (Poppenberger et al., 2003). Other DON derivatives such as 3Ac-DON, 15Ac-DON and DOM-1 show wide variability in their toxicity profiles. Different studies have concurred that 3Ac-DON and DOM-1 are less toxicologically potent than DON, whilst 15Ac-DON is usually more potent, albeit depending on the endpoint of interest (Payros et al., 2016).

Observations of reduced toxicological activity, similar to DON conjugates, have also been documented in *in vitro* assays for derivatives of ZEN. Both ZEN14Glc and ZEN16Glc were tested in Caco-2 cells for cytotoxicity and found to be negative (Cirlini et al., 2016). ZEN14Glc has been demonstrated to exhibit reduced ER binding capacity compared to ZEN (Poppenberger et al., 2006), a fact that was recently confirmed by a mixed *in vitro*/*in silico* approach confirming its inability to effectively bind and activate ERs (Dellafiora et al., 2017). *In vitro* and *in silico* modelling also showed that ZEN14Sulf did not activate ERs and ZEN14Sulf was non-oestrogenic in MCF-7 human breast cancer cells (Drzymala et al., 2015). Toxicity studies on other modified mycotoxins, apart from those previously discussed in Section 2.1, have been scarce.

Although conjugations of mycotoxins with various moieties appear to mostly alleviate their toxicological activity, possible health effects can still be manifested from the liberation of toxic aglycones during mammalian digestion. Evidence from *in vitro* digestive systems strongly suggests that several important modified trichothecenes (DON3Glc, NIV3Glc, T2-Glc and HT2-3-Glc) and modified ZEN compounds such as ZEN14Glc, ZEN14Sulf, α-zearalenol-14-glucoside (α-ZEL14Glc) and β-zearalenol-14-glucoside (β-ZEL14Glc) remain unaffected under conditions prevailing in the upper GIT (Gratz, 2017). Several modified trichothecenes, namely DON3Glc, NIV3Glc and T2-Glc, have been found to not be efficiently transported through intestinal epithelial cell monolayers, whilst their precursors were able to be transferred to various degrees (De Nijs et al., 2012; Gratz et al., 2017). For ZEN14Glc, findings are somewhat in disagreement, with one study reporting no transport across intestinal epithelium monolayers and no significant release of ZEN after 24 h of exposure to ZEN14Glc (2 μM), whereas another study reported uptake by Caco-2 cells, as well as de-glucosylation of approximately 20% of the applied 40 μM ZEN14Glc, being released as apical ZEN (Cirlini et al., 2016). Importantly, all these modified forms can be effectively de-conjugated by

human colonic microbiota with varying kinetics, and further metabolised to many currently unknown metabolites (Berthiller et al., 2011; Dall'Erta et al., 2013; Gratz et al., 2017).

Complementary to *in vitro* data, *in vivo* studies have also compared the digestive fate and toxic responses of modified mycotoxins with their native analogues. In an *in vivo* metabolism experiment, equimolar amounts of DON and DON3Glc were orally administered as single doses in six Sprague-Dawley male rats (Nagl et al., 2012). Most of the applied DON3Glc was recovered in faeces as DON or DOM-1, indicating extensive de-conjugation, presumably in the GIT of rats. Interestingly, intestinal absorption of DON3Glc was measured to be reduced by a factor of four compared to DON. The *in vitro* stability of DON3Glc in the stomach was verified *in vivo* by Veršilovskis et al. (2012), who administered by gavage 25 µg of DON3Glc to two male Wistar rats and was able to detect only ca 2% of DON in the stomach. However, the same study documented an extensive de-conjugation of DON3Glc in the small intestine attributable to the enzymatic activity of tissue β-glucosidases. Nagl et al. (2014), also conducted a follow-up study in which equimolar amounts of DON and DON3Glc were orally administered to four male piglets on days 3 and 9 of the study, respectively, and DON3Glc intravenously on day 13. The largest proportion of DON3Glc was excreted in urine and only negligible amounts were found in faeces, revealing species-specific differences in the metabolism of this modified mycotoxin. Nonetheless, the fact that DON3Glc was almost completely absent from pig excreta is line with results obtained for rats. *In vivo*, DON3Glc was also found incapable of eliciting pro-inflammatory cytokine and chemokine messenger RNA (mRNA) responses in mice following oral exposure via gavage to 2.5 mg DON3Glc/kg bw (Wu et al., 2014a). At similar dose levels (2.5–10 mg/kg bw), DON3Glc rapidly elicited anorectic responses that mimicked DON in a nocturnal mouse food consumption study but was markedly less effective at inducing emesis in minks (Wu et al., 2014b). Taken together, available data on DON3Glc suggest that this modified mycotoxin is only absorbed in the GIT, and release of the aglycone only takes place in distal parts of the intestine, thus minimising the absorption of free DON. It is also significantly less toxicologically active compared to its precursor.

However, the first investigation concerning the *in vivo* fate of a glucosylated mycotoxin, was conducted by Gareis et al. (1990). A diet artificially contaminated with synthesised ZEN14Glc (396 µg/kg) was fed to a female pig over a period of two weeks. Analysis of the excreta revealed considerable amounts of ZEN, α-ZEL and β-ZEL, but no presence of the initially dosed modified mycotoxin. The authors thus posited complete de-conjugation of ZEN14Glc during digestion and release of the aglycone ZEN, which was then further metabolised. Notably, no clinical signs of hyperoestrogenism were observed in the animal. In a rat uterus bioenlargement bioassay, six Sprague-Dawley rats per group were administered intragastrically equimolar amounts (630 nmol) of either ZEN or ZEN14Sulf (Plasencia and Mirocha, 1991). The results indicated that ZEN14Sulf partially

retained the oestrogenic properties of ZEN. Hydrolysis of ZEN14Glc to free ZEN in the stomach of rats has been reported, in contrast to DON3Glc, which remained stable. After administration of 25 µg ZEN14Glc to two Wistar rats, considerable amounts of ZEN (approx. 18% of the administered dose) were found in the stomach, indicating hydrolysis of the modified mycotoxin in the upper digestive tract (Veršilovskis et al., 2012). In another *in vivo* experiment, ZEN (10 µg/kg bw) and equimolar amounts of ZEN14Glc, ZEN16Glc and ZEN14Sulf were orally administered to pigs as a single bolus using a repeated measures design (Binder et al., 2017). All modified forms were readily hydrolysed to ZEN and converted to other mostly unknown metabolites in the GIT of pigs. Of significance is that glucosylated metabolites of ZEN were recovered to a greater extent in faeces, denoting reduced bioavailability and slightly reduced toxicity. The most recent study exploring the metabolic fate of ZEN, α-ZEL, β-ZEL, ZEN14Glc and ZENSulf in pigs, reported age-dependent differences in the oral bioavailability of ZEN (ranging between 61–85%), complete absorption of α-ZEL and β-ZEL, complete hydrolysis of both ZEN14Glc and ZEN14Sulf to ZEN and extensive glucuronidation of all compounds (Catteuw et al., 2019). On the basis of their findings, the authors highlighted the importance of including modified ZEN forms in feed analysis and utilising glucuronide metabolites of ZEN derivatives in biomonitoring studies.

To summarise, from the limited existing toxicity data on a handful of modified mycotoxins that have been reasonably toxicologically tested, two generalised conclusions can be drawn. Firstly, from *in silico*, *in vitro* and *in vivo* studies, the few currently known modified mycotoxins appear to possess (considerably) reduced intrinsic toxicity compared to their native analogues. However, the toxicological relevance of certain compounds ought not be underestimated, as they may act via different, as yet obscure mechanisms compared to their precursors. Secondly, the bioavailability and digestive fate of modified forms can significantly differ among them and from their precursors, leading to the assumption that these compounds cannot be viewed as a homogeneous group of compounds with identical toxicological characteristics. Finally, the toxicological implications associated with the release of an aglycone in the lower GIT warrants further investigations to better assess the impact on intestinal cells (histomorphology, barrier function and immune responses), potential colonic absorption and the impact on the gut microbial composition and eventual potential systemic manifestation in an organism.

2.4 Analysis of mycotoxins and derivatives

Whenever food/feed safety risk assessments of mycotoxins and their derivatives result in unfavourable outcomes, regulatory measures, e.g. in the form of maximum levels, may ensue. Devising appropriate risk management strategies often involves complex

processes, heavily dependent on the evaluation of occurrence, exposure and toxicity data for the contaminants under investigation (EFSA, 2012). To obtain ample qualitative and quantitative data on these toxins in a reliable manner, the development of fit-for-purpose analytical methods is necessary. Additionally, routine surveillance of regulated mycotoxins requires robust, highly-sensitive detection methods, allowing for the determination of a multitude of target compounds, i.e. known analytes of interest in various sample matrices. Native mycotoxins in food- and feedstuffs are commonly analysed by chromatographic methods such as thin layer chromatography (TLC), gas chromatography (GC), LC, or rapid immunochemical methods e.g. enzyme-linked immunosorbent assay (ELISA).

For the majority of analytical applications that require reliable and accurate quantification, LC–MS is nowadays recognised as the go-to method by both research and official control laboratories. Native and modified mycotoxin determination with hyphenated chromatographic methods is frequently a multistep process, including sampling, homogenisation, extraction, clean-up and, when needed, analyte enrichment prior to determination (Cirlini et al., 2012). The application of matrix-specific sample preparation techniques and optimisation of chromatographic and MS parameters are both essential for sensitive and accurate concurrent analysis of a wide range of chemically diverse mycotoxins. These subjects are in the focus of this section and especially as regards LC–MS methodologies.

2.4.1 Sampling and sample preparation

Analytical procedures for mycotoxin determination ordinarily commence with sampling and sample comminution to obtain a suitable size that would allow the extraction of analytes and subsequent measurements. The aim of sampling is to collect representative sample(s), e.g. from a lot of an agricultural product, of a workable size in the laboratory (Köppen et al., 2010). Due to the heterogeneous distribution of mycotoxins in agricultural commodities, adequate sampling is a very crucial step for reliable analytical determination. Generally, the most effective approaches to reduce the variability of mycotoxin test procedures are to increase the laboratory sample size, the degree of sample comminution and the number of aliquots quantified (Whitaker, 2006). The methods of sampling and analysis for official control of the levels of mycotoxins in foodstuffs are laid down in Commission Regulation (EC) No 401/2006 (EC, 2006a). Strategies applied for sampling of parent mycotoxins are also suitable for conjugated mycotoxins (EFSA, 2014). For research purposes, sampling approaches may have to be considerably adjusted in order to embrace the experimental hypothesis and conditions (sample amount, type, chemical stability of analytes, etc.). An additional complication in certain metabolomics studies is that during sampling, immediate quenching of all

metabolic processes should be performed without alteration of the metabolic state (Kluger et al., 2015b). Storage conditions must also not permit alterations of the metabolic state or cause changes to metabolite species and levels.

Sample preparation is the next key step for a successful protocol in the chemical analysis of mycotoxins. The majority of LC–MS methods rely on the extraction of analytes from the biological matrices into a liquid phase and optional clean-up to remove undesirable matrix components (Turner et al., 2009). The extraction methods applied depend on the chemistry of the target analytes and on the type of matrix for which extraction is performed. Because of the wide range of physico-chemical properties of mycotoxins, particular consideration needs to be given to the extraction efficiency of the solvent mixture. Conjugated mycotoxin derivatives are usually more polar than their precursors. Therefore, extraction schemes should facilitate the removal of both forms from sample matrices with comparable recoveries. Several different extraction procedures are used, of which the oldest, but still frequently used, is solvent extraction (Kralj Cigić and Prosen, 2009). Typically, the extraction of polar analytes is favoured by the presence of water in the extraction solution mixture, whilst the extraction of hydrophobic mycotoxins is enhanced by organic solvents. The most widely used extraction solvents are mixtures of methanol:water and acetonitrile:water in varying ratios, regularly containing modifiers such as acids or bases. (Meneely et al., 2011). According to the literature, the extraction of DON, NIV, HT2, T2 and ZEN, as well as many of their derivatives, has been successfully achieved using various ratios of acetonitrile and water, acidified with formic or acetic acid (Zachariasova et al., 2010; De Boevre et al., 2012; Malachová et al., 2014). In a metabolomics study, Cajka et al. (2014) accomplished optimal extraction of polar/medium polar barley metabolites employing either acetonitrile:water (84:16, v/v) or a mixture of methanol:water (50:50, v/v). The authors eventually chose the latter solution due to its broadest representation of metabolites based on polarity. Other extraction techniques that have been successfully incorporated in mycotoxin analysis include accelerated solvent extraction, pressurised liquid extraction, microwave-assisted extraction and supercritical fluid extraction (Krska et al., 2008).

For further purification and analyte enrichment, liquid extracts can be submitted to solid phase extraction (SPE), specifically tailored multifunctional columns (e.g. MycoSep® push-through clean-up columns), immunoaffinity columns or modifications of the "quick, easy, cheap, effective, rugged and safe" (QuEChERS) technique (Malachová et al., 2017). The purpose of sample clean-up is to enhance the signal-to-noise ratio and minimise interference by matrix components that may cause analyte signal suppression or enhancement (SSE; i.e. matrix effects) (Trufelli et al., 2011). The majority of sample preparation techniques used in mycotoxin analyses have been developed for the determination of parent mycotoxins, and more so the regulated ones (see Section 2.7). As a consequence, they may not be suitable for the determination of modified

mycotoxins. Vendl et al. (2009), evaluated the suitability of several clean-up strategies (C_{18}-SPE, primary and secondary amines, MycoSep and immunoaffinity columns) for the determination of DON, ZEN and their major modified metabolites in cereals and cereal-derived products by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Overall, none of the tested clean-up methods proved suitable to accommodate a wide range of polarities and the final method thus used no sample clean-up.

The limited availability of analytical standards for modified mycotoxins has led to the development of indirect methods as an attractive alternative to target analysis. Such approaches are based on chemical or enzymatic treatments that cleave the conjugated moieties and release the parent mycotoxin. The supposed advantage of these techniques is that measurements of all modified forms present within a sample can be performed without the need for analytical standards, because the modified mycotoxin concentration is estimated as the total precursor analyte content after subtracting the free precursor content. However, a critical assessment was conducted of three indirect methods for total DON determination, and none was found able to de-conjugate the parent mycotoxin (DON) from the modified forms DON3Glc, 3Ac-DON and 15Ac-DON (Malachová et al., 2015). The authors concluded that use of acidic or alkaline hydrolytic process was not suitable for the indirect determination of DON and efforts should be directed towards methods relying on enzymatic hydrolysis. Ideally, accurate determination of modified forms should be based on analytical standards.

2.4.2 Liquid chromatography–mass spectrometry

LC coupled with ultraviolet, diode array or fluorescence detectors have been widely used techniques for reliable and robust detection of mycotoxins in food at reasonably low levels. Over the past two decades, however, LC–MS/MS based methods have been established as the "golden standard" for determination of low-molecular weight food contaminants, including mycotoxins, over a concentration range of several orders of magnitude (Righetti et al., 2016). Key features of LC–MS/MS methods are their ability for multi-analyte determination with high analytical selectivity, sensitivity, accuracy and robustness (Songsermsakul and Razzazi-Fazeli, 2008). These techniques also offer highly specific chemical information on the molecular mass and the isotopic fine structure of compounds, making them extremely useful in metabolomics studies for identification and characterisation of modified mycotoxins (De Boevre et al., 2015).

LC is a versatile technique for the separation of analytes in a mixture, according to their physico-chemical properties, by chemical interactions of analytes between a liquid mobile phase and a solid stationary phase. Chromatographic separation in multi-mycotoxin methods is achieved either with high performance liquid chromatography (HPLC) instruments or with the more modern technology, ultra high performance liquid

chromatography (UHPLC). In modern LC–MS/MS applications, chromatographic separation is mainly used to attenuate ion suppression effects, frequently occurring when multiple compounds reach the ion source simultaneously. Additionally, in metabolomics experiments in particular, chromatography can be used as retention-time identifier for the analyte of interest (Patti, 2011). Reversed phase which is considered as the most popular type of LC separation in mycotoxin analyses is relying on hydrophobic interactions between the analytes and the stationary phase for separation, ensuring retention for a wide range of substances. The eluents in reversed phase chromatography are polar (e.g. mixtures of water with acetonitrile or methanol), whilst the column materials are apolar (most often C_{18}) (Snyder et al., 2010). Hydrophilic interaction chromatography (HILIC), which is a variant of normal phase LC, is ideal for separation of small polar compounds (Buszewski and Noga, 2012).

Following chromatographic separation, analytes are transferred from the liquid phase to the gaseous phase and are ionised before they enter the mass spectrometer, which operates under high vacuum in order for the generated ions to reach the detector intact. Different MS interfaces have been utilised for analysis of mycotoxins in food and feed, as well as in biological matrices (e.g. organ tissues, urine, faeces, blood and other samples). Nowadays, modern LC–MS instruments utilise atmospheric pressure chemical ionisation (APCI), atmospheric pressure photo-ionisation (APPI) or electrospray ionisation (ESI) interfaces (Songsermsakul and Razzazi-Fazeli, 2008). ESI is the most widely utilised ionisation technique in LC–MS/MS applications and belongs to the so-called "soft" ionisation techniques in which ions are generated without decomposition of the analyte. In positive mode, ESI leads to the formation of protonated ions $[M+H]^+$, or adducts such as $[M+Na]^+$. In the negative mode, ESI deprotonated ions $[M–H]^–$ or adducts, e.g. $[M+CH_3COO]^–$.

Mass spectrometers are then able to separate charged particles based on their mass-to-charge ratio (*m/z*) and measure relative abundances of ions (Boyd et al., 2011). Most quantitative methods used nowadays rely on LC–MS instrumentation and particularly triple quadrupole (TQ) mass spectrometers. These provide a high linear range and good sensitivity, but on the downside, they require tuning of the analytes during method development and can only be used for measurement of already known analytes. With this instrument, selected reaction monitoring (SRM) mode is possible, which is highly capable for sensitive quantitation (Holčapek et al., 2012). In the SRM mode, the first and the third quadrupoles act as mass filters, whilst the second one is the collision chamber, in which the target ion is fragmented. The ions are fragmented by collision-induced dissociation with a collision gas (usually nitrogen or argon) to form product ions that are further detected. The MS data are displayed in the total ion chromatogram (TIC), which is created by summing up intensities of all mass spectra belonging to the same scan. In the extracted ion chromatogram (EIC) each selected mass or mass transition is shown separately. Ion trap and time-of-flight (TOF) are two also commonly used in mycotoxin

analytics. QTOFs are hybrid HRMS instruments, combining a quadrupole with a TOF and are capable for measurements of high mass accuracy in both full and MS/MS modes (Holčapek et al., 2012).

Hyphenated chromatographic techniques, and particularly LC–MS/MS, allow for rapid, sensitive and accurate quantification that fosters the strong tendency of recent years towards development of multi-(modified) mycotoxin determinations (Cirlini et al., 2012). In 2006, a method was developed for the determination of 39 free and modified mycotoxins in two consecutive chromatographic runs (ESI^- and ESI^+) (Sulyok et al., 2006). A few years later, an LC–MS/MS method using both ESI^- and APCI probed was published that could analyse simultaneously DON and ZEN, along with eight of their modified forms (Vendl et al., 2009). Multi-methods have been further expanded, fully exploiting the power offered by MS detection relying on simplified sample preparation processes that can be applied without necessitating dedicated sample clean-up. Nowadays LC–MS/MS methods are able to accommodate accurate and rapid determination of hundreds of analytes in a single run and in different matrices (Malachová et al., 2014).

In today's world, however, with more and more toxic substances constantly receiving regulatory attention, the use of HRMS has unlocked additional capabilities in mycotoxin research, including concurrent identification of theoretically unlimited non-target compounds, even at minute concentrations, as well as retrospective data analysis (Kluger et al., 2015a). The use of full scan acquisition mode with high sensitivity, combined with high resolving power (up to 100,000 full width at half maximum, FWHM; defined at *m/z*) and accurate mass measurement (< 5 ppm) are the main characteristics of the increased popularity of HRMS. The mass accuracy of a measurement is the relative difference between the measured mass and the exact mass (i.e. sum of the most abundant isotopes of the constituent atoms of this molecule; Murray et al., 2013), expressed in parts per million (ppm) (De Boevre et al., 2015). The accurate mass of a precursor ion, spectral information on elemental composition, together with the accurate mass of its fragment ions are valuable pieces of information in the identification of its chemical structure. Although such data are crucial in metabolite identification, and can relatively easily be obtained with HRMS apparatus, locating metabolites of interest can be a daunting task in reality. To address this issue, targeted and untargeted metabolomics approaches, as well as sophisticated data processing algorithms and software, which can detect and process metabolite-derived MS signals and chromatographic peaks within huge amounts of LC–HRMS raw data, have been developed (see Section 2.6).

In LC–MS/MS analyses, however, SSE caused by matrix components is a major drawback, which can impair accuracy and sensitivity of a method. Especially in analytics where no sample clean-up is applied, detection of low-level analytes may severely be hampered by co-eluting components that alter the ionisation efficiency (Gosetti et al., 2010). Consequently, matrix effects can directly influence the amount of analyte ions

reaching the detector, causing either lower (suppression) or higher (enhancement) analyte signal compared to that obtained from the same compounds in neat standard solution, which in turn will lead to underestimation or overestimation of target analyte concentrations. The exact mechanism behind matrix effects remains unknown, but it has been hypothesised that matrix components might be interfering with the process of analyte droplet formation and transition to the gaseous phase or creating competition with target compounds for the limited available charges inside the ionisation interface (Taylor, 2005). Matrix effects are substance-dependent, and the chemical nature of different mycotoxins has a significant effect on the extent of matrix effects (Malachová et al., 2017). The type of matrix analysed, the sample preparation, as well as the chromatographic and MS conditions can also greatly affect the degree of matrix effects (Gosetti et al., 2010).

There are, however, strategies to overcome matrix effects and at least partially compensate for their negative influence in LC–MS/MS methods. But first, it is crucial to determine whether matrix effects are present and to what extent. Matrix induced SSE can be estimated by the equation proposed by Sulyok et al. (2006). For instance, sample dilution to minimise undesired matrix effects or clean-up techniques to remove interfering matrix components are probably the most widely used approaches in mycotoxin analysis (Krska et al., 2008). Although, dilution might not be appropriate if target analytes are expected to be present in trace amounts and sample clean-up techniques are frequently not applicable to a multitude of chemically distinct mycotoxins and are time-consuming, as previously discussed. If matrix effects cannot be eliminated with sample preparation, adjustments to the chromatographic conditions and/or alternate calibration methods should be considered (Trufelli et al., 2011). An attractive alternative to time-consuming manual sample preparation techniques that can minimise the effect of interfering matrix components, without the need for manual sample preparation is on-line sample clean-up coupled with LC–HRMS (Ates et al., 2014). Another possible approach to cope with matrix effects is the stable isotope dilution assay (SIDA). Several multi-mycotoxin methods have been developed based on this technique that use labelled isotopologue standards (Varga et al., 2012; Zhang et al., 2017), including also isotopologues of modified forms (DON3Glc, 3Ac-DON and 15Ac-DON) (Habler and Rychlik, 2016).

2.4.3 Method validation and performance characteristics

Method validation is a crucial process in the development of any new analytical method. Its purpose is to demonstrate that the method is suitable for the intended applications and it is capable of providing reliable results. However, of the multitude of methods that have been published over the years regarding the determination of mycotoxins in food and feed, only a limited number include performance characteristics and in-house

validation data (Krska et al., 2008). In the EU, the general guidelines on the performance of analytical methods and the interpretation of results are laid down in Commission Decision 2002/657/EC (EC, 2002a), which defines validation as: "the confirmation by examination and the provision of effective evidence that the particular requirements of a specific intended use are fulfilled." Such evidence, or functional qualities attributed to an analytical method, are determined via several performance characteristics including specificity, linearity, accuracy, trueness, recovery, precision, repeatability, reproducibility, ruggedness and the detection limit. It should be noted that there is no existing regulatory framework for the proper validation of multi-mycotoxin methods or confirmatory methods used for the identification of unknown compounds.

An overview of the typical performance characteristics evaluated during the validation of methods used in the analysis of mycotoxins is provided in Malachová et al. (2017). Briefly, method specificity describes the ability to distinguish between a target analyte and other components under the experimental conditions (e.g. isomers, impurities and matrix). The linearity of an analytical procedure is another important performance characteristic that denotes its ability to obtain test results (within a given working range) that are directly proportional to the concentration of the analyte in the sample. The accuracy of a quantitative method refers to the closeness of agreement between a test result and the accepted reference value and is determined through trueness and precision. The verification of trueness is accomplished by analysing certified reference materials or, if not available, by measuring the recovery (i.e. percentage of the true concentration of a substance) of additions of known analyte amounts to a "blank" matrix. The precision of a method is the degree of scatter between a series of measurements and is expressed as the relative standard deviation (RSD) of the repeated analysis of a reference or fortified material under the prescribed conditions. Hence, repeatability is the precision under the same operating conditions over a short interval of time (e.g. repeatability, RSD_r). On the other hand, reproducibility can be defined either as within-laboratory precision by estimating the variation of measurements between different days, different analysts, etc. (e.g. precision, RSD_R) or as precision between laboratories. Lastly, the limit of detection (LOD) is defined as the lowest concentration of analyte in a sample that can be discerned from a blank sample with a specific certainty, whereas the limit of quantification (LOQ) is the lowest concentration at which the analyte can be determined with a certain precision and accuracy.

2.5 Natural occurrence of modified *Fusarium* mycotoxins

Unlike humans and animals, which are able to metabolise mycotoxins and eliminate water-soluble metabolites through urine and faeces, *in planta*-formed metabolites must be expected to remain in the infested plant and could reach consumers via the consumption of food items containing the contaminated plant material (EFSA, 2017a). Survey data concerning the mycotoxin content in different commodities are of considerable importance for establishing appropriate risk management measurers and devising mitigation strategies. To date, analytical methodologies have mainly focused on investigation of the natural presence of parent mycotoxins in foods and feeds, whilst limited data are available on the presence of modified forms. An overview of known modified DON, NIV, HT2, T2 and ZEN mycotoxins, generated in plants and/or fungi, together with the origin and source of initial documentation is given Table 1.

Several survey studies have been conducted regarding the natural occurrence of DON3Glc in cereal crops and cereal-derived goods, including an in-depth review with extensive occurrence data that was recently published in the format of a scientific opinion by (EFSA, 2017b). Briefly, it was estimated that the ratios of concentrations of 3Ac-DON to DON, 15Ac-DON to DON and DON3Glc to DON were 10%, 15% and 20%, respectively. The highest mean concentrations of DON and the sum of DON, 3Ac-DON, 15Ac-DON and DON3Glc were recorded for food in the category of "Products for special nutritional use" and for feed, in "Cereal straw", with their co-occurrence varying considerably between different food, feed and grain categories. DON3Glc, in particular, has been reported in barley, oats, wheat and maize, as well as the cereal-derived products malt, beer and bread (Crews and MacDonald, 2016). 3Ac-DON and 15Ac-DON are also consistently abundant in a large proportion of cereal grains and commodities (breakfast cereals, fibre-enriched bread and oatmeal, among others) (De Boevre et al., 2013; Bryła et al., 2016).

Table 1. Overview of known modified forms of DON, NIV, HT2, T2 and ZEN in plants and/or fungi. Compounds identified in the course of this thesis have not been included.

Origin	Modified mycotoxin (precursor)	Producing organism(s)	Reference(s)
Plants and fungi	DON3Glc (DON)	maize cell suspension cultures; *Trichoderma* strains	Sewald et al., 1992; Tian et al., 2016
	HT2-3-Glc (HT2, T2)	wheat and oat kernels; *Fusarium sporotrichioides* (unconfirmed)	Busman et al., 2011; Latanzzio et al., 2012
	T2-Glc (T2)	wheat and oat kernels; yeasts of the *Trichomonascus* clade	Busman et al., 2011; McCormick et al., 2012
	3Ac-T2 (T2)	*Fusarium graminearum*, *Fusarium nivale*; oats (artificially inoculated)	Yoshizawa et al., 1980; Meng-Reiterer et al., 2016
	ZEN14Glc (ZEN)	*Rhizopus* spp.; maize cells	Kamimura, 1986; Engelhardt et al., 1988
	ZEN16Glc (ZEN)	barley seedlings, wheat and *Brachypodium distachyon* cell suspension cultures; *Aspergillus oryzae*, *Rhizopus* spp.	Kovalsky Paris et al., 2014; Brodehl, 2014
	ZEN14Sulf (ZEN)	*Fusarium graminearum*; barley, maize, oats, wheat	Plasencia and Mirocha, 1991; De Boevre et al., 2012
	α-ZEL (ZEN)	*Fusarium roseum*; maize cells	Hagler et al., 1979; Engelhardt et al., 1988
	β-ZEL (ZEN)	*Fusarium roseum*; maize cells	Hagler et al., 1979; Engelhardt et al., 1988
	α-ZEL14Glc (ZEN)	maize cells; *Aspergillus oryzae*, *Rhizopus* spp.	Berthiller et al., 2009; Brodehl, 2014
	β-ZEL14Glc (ZEN)	maize cells; *Aspergillus oryzae*, *Rhizopus* spp.	Berthiller et al., 2009; Brodehl, 2014
Fungi	3Ac-DON (DON)	*Fusarium roseum*	Yoshizawa and Morooka, 1973
	15Ac-DON (DON)	*Fusarium graminearum*	Miller et al., 1983
	neosolaniol (HT2, T2)	*Fusarium* (unidentified species)	Torp and Langseth, 1999
	T2-triol (HT2, T2)	*Fusarium* (unidentified species)	Torp and Langseth, 1999
	T2-tetraol (HT2, T2)	*Fusarium* (unidentified species)	Torp and Langseth, 1999
	zearalanone (ZEN)	*Saccharomyces cerevisiae*	El-Sharkawy and Abul-Hajj, 1988
	α-zearalanone (ZEN)	*Fusarium* (unidentified species)	Erasmuson et al., 1994
	β-zearalanone (ZEN)	*Fusarium* (unidentified species)	Erasmuson et al., 1994
Plants	DON-di-glucoside (DON)	barley, malt, commercially baked cereal goods	Zachariasova et al., 2012
	DON-tri-glucoside (DON)	barley, malt, commercially baked cereal goods	Zachariasova et al., 2012
	DON-tetra-glucoside (DON)	barley, malt, commercially baked cereal goods	Zachariasova et al., 2012
	DON-hexitol (DON)	wheat (artificially inoculated)	Kluger et al., 2015b
	DON-S-cysteine (DON)	wheat (artificially inoculated)	Kluger et al., 2015b
	DON-S-cysteinyl-glycine (DON)	wheat (artificially inoculated)	Kluger et al., 2015b

Table 1. Continued.

Plants	DON-glutathione (DON)	wheat (artificially inoculated)	Kluger et al., 2015b
	DON-di-hexoside (DON)	wheat (artificially inoculated)	Kluger et al., 2015b
	DON-anhydro-glutathione (DON)	wheat (artificially inoculated)	Kluger et al., 2015b
	DON-malonyl-glucoside (DON)	wheat (artificially inoculated)	Kluger et al., 2015b
	15-acetyl-DON-3-glucoside (DON)	wheat (artificially inoculated)	Kluger et al., 2015b
	DON-3-sulfate (DON)	wheat (artificially inoculated)	Warth et al., 2015
	DON-15-sulfate (DON)	wheat (artificially inoculated)	Warth et al., 2015
	NIV3Glc (NIV)	wheat (artificially inoculated)	Nakagawa et al., 2011
	HT2-di-glucoside (HT2, T2)	barley	Veprikova et. al., 2012
	T2-di-glucoside (T2)	corn	Nakagawa et al., 2013
	15-acetyl-T2-tetraol-glucoside (HT2, T2)	oats (artificially inoculated)	Meng-Reiterer et al., 2016
	dehydro-15-acetyl-T2-tetraol-glucoside (HT2, T2)	oats (artificially inoculated)	Meng-Reiterer et al., 2016
	hydroxy-HT2-hexosyl-glucoside (HT2, T2)	oats (artificially inoculated)	Meng-Reiterer et al., 2016
	hydroxy-HT2-glucoside (HT2, T2)	oats (artificially inoculated)	Meng-Reiterer et al., 2016
	hydroxy-HT2 (HT2, T2)	oats (artificially inoculated)	Meng-Reiterer et al., 2016
	hydroxy-HT2-anhydro-hexosyl-glucoside (HT2, T2)	oats (artificially inoculated)	Meng-Reiterer et al., 2016
	T2-triol-glucoside (HT2, T2)	oats (artificially inoculated)	Meng-Reiterer et al., 2016
	dehydro-HT2-glucoside (HT2, T2)	oats (artificially inoculated)	Meng-Reiterer et al., 2016
	HT2-hexosyl-glucoside (HT2, T2)	oats (artificially inoculated)	Meng-Reiterer et al., 2016
	HT2-malyl-glucoside (HT2, T2)	oats (artificially inoculated)	Meng-Reiterer et al., 2016
	HT2-malonyl-glucoside (HT2, T2)	oats (artificially inoculated)	Meng-Reiterer et al., 2016
	HT2-anhydro-hexosyl-glucoside (HT2, T2)	oats (artificially inoculated)	Meng-Reiterer et al., 2016
	feruloyl-T2 (T2)	oats (artificially inoculated)	Meng-Reiterer et al., 2016
	ZEN-di-hexoside (ZEN)	*Arabidopsis thaliana*	Berthiller et al., 2006
	α-ZEL-di-hexoside (ZEN)	*Arabidopsis thaliana*	Berthiller et al., 2006
	β-ZEL-di-hexoside (ZEN)	*Arabidopsis thaliana*	Berthiller et al., 2006
	ZEN-malonyl-hexoside (ZEN)	*Arabidopsis thaliana*	Berthiller et al., 2006
	α-ZEL-malonyl-hexoside (ZEN)	*Arabidopsis thaliana*	Berthiller et al., 2006
	β-ZEL-malonyl-hexoside (ZEN)	*Arabidopsis thaliana*	Berthiller et al., 2006
	ZEN-pentosyl-hexoside (ZEN)	*Arabidopsis thaliana*	Berthiller et al., 2006
	α-ZEL-pentosyl-hexoside (ZEN)	*Arabidopsis thaliana*	Berthiller et al., 2006
	β-ZEL-pentosyl-hexoside (ZEN)	*Arabidopsis thaliana*	Berthiller et al., 2006

Various modified forms of HT2 and T2 have also been described in the literature, as a result of fungal and/or plant metabolism. In an early study, *Baccharis* spp. were found able to metabolise T2 into the phase I and II metabolites HT2, 3'-hydroxy-HT2 and T2-tetraol (Mirocha et al., 1988). Similarly, yeasts have been reported capable of biotransforming HT2 and T2. For instance, yeasts of the *Trichomonascus* clade biotransformed T2 into 3Ac-T2, T2-Glc and neosolaniol (McCormick et al., 2012). *In planta* glucosylation was documented by Busman et al., (2011), who detected and tentatively identified HT2- and T2-3-glucosides in oat and wheat kernels inoculated with *F. sporotrichioides*. This finding was confirmed by the detection of these compounds in naturally contaminated oats and wheat (Lattanzio et al., 2012), as well as in commercially available maize powder reference material (Nakagawa et al., 2012). These developments were followed by the discovery of di-glucosylated forms in naturally contaminated cereal grains (Veprikova et al., 2012; Nakagawa et al., 2013). Notably, accurate mass measurements and the fragmentation pattern provided evidence, in both studies, for possible glucosylation of HT2 at the C-3 and C-4 positions. The ubiquitous prevalence of both HT2 and T2 mono-glucosides in more than 75% of 20 naturally contaminated samples of barley, oats and wheat was confirmed, and the presence of two HT2-di-glucoside isomers in barley (Veprikova et al., 2012). As no reference standards for these modified forms were available at the time, the authors reported that the mean peak area ratios (%) between glucosylated to parent toxins were around 17%. During a 2015 study, HT2-3-Glc was quantified at levels of up to 163 µg/kg in 17 out of 18 field barley samples, whereas T2-Glc was detected in only a few samples and at low concentrations (Lattanzio et al., 2015). In a survey study, 30 samples from a variety of cereal-derived food and feed matrices were collected from the Belgian market and analysed, but HT2-3-Glc and T2-Glc could not be detected, even though both parent forms were present in some of the samples (De Boevre et al., 2012).

The only *in planta* conjugated metabolite of NIV that has been characterised to date is its 3-glucosylated form, which was first detected in wheat grains artificially inoculated with *F. graminearum* (Nakagawa et al., 2011) and then structurally identified as NIV3Glc by LC–MS/MS and nuclear magnetic resonance (NMR) measurements (Yoshinari et al., 2014). In the former study, more than 15% of NIV was estimated to have been converted into its glucoside, whereas in the latter study, NIV3Glc was quantified in naturally contaminated wheat, at concentrations up to 4000 µg/kg and accounting for up to 27% of the total parent toxin. In a very recent survey in Polish wheat grains (n = 300), NIV3Glc was determined in approximately 15% of the samples collected in 2017 and 2018, at concentrations ranging between 5–40 µg/kg (Bryła et al., 2019). The NIV3Glc/NIV molar ratio was between 15–20%. Even though NIV3Glc is the only currently known phase II metabolite of NIV, other conjugated metabolites are likely to be formed due to the presence of four –OH groups in its backbone.

Modified forms of ZEN have been described in mammals, plants and fungi following biotransformation of native ZEN, with a significant number of modified ZEN compounds being common among different species (EFSA, 2016). Hitherto, only a few studies have described the presence of ZEN modified forms in crop plants. Schneweis et al. (2002), reported that ZEN was present in 92% of the 24 field wheat samples from a 1999 harvest in Bavaria, with levels ranging between 11 to 860 µg/kg. ZEN14Glc was detected in 42% of the samples analysed at concentrations between 17–104 µg/kg, all of which were ZEN-positive and exhibited a linear concentration correlation between the two compounds. In a survey of ZEN and its derivatives in 84 cereal-based foods, ZEN14Sulf predominated in abundance, with the highest quantity being about 6 µg/kg; none of the analysed samples were found to contain any ZEN14Glc, α- and β-ZEL or α- and β-ZEL14Glc (Vendl et al., 2010). The 2012 Belgian market survey reported the occurrence of ZEN and a number of its modified forms in cereal-based food products (De Boevre et al., 2012). The findings of that study revealed that α- and β-ZEL occurred in 53% and 63%, respectively, of the samples analysed (n = 30). A noteworthy result was that in one maize sample, the sum of ZEN14Glc, ZEN14Sulf and β-ZEL14Glc, in comparison to the concentration of ZEN in the same sample, indicated that approximately 90% of the available precursor mycotoxin had been metabolised. Lastly, Brodehl et al. (2014) described the transformation of more than 50% ZEN into ZEN14Glc, ZEN16Glc, α-ZEL and the novel metabolite α-zearalenol-sulfate by *Rhizopus* and *Aspergillus* species, both commonly used species in the biotechnological production of fermented food (e.g. soy products). In general, whole grain cereal products appear to be the source of ZEN14Glc, α-ZEL14Glc, β-ZEL14Glc and ZEN14Sulf in food and feed, and modified forms may account for a few per cent up to 100% relative to ZEN (EFSA, 2016).

Natural occurrence data on modified *Fusarium* mycotoxins, both in grains and products thereof, are becoming more widely available. Currently, most prevalence data exist for DON3Glc, 3Ac-DON, 15Ac-DON and a few modified ZEN forms. The data clearly reflect the ubiquitous presence of modified mycotoxins in most cereal crops and cereal-derived products, thus justifying the research efforts in the field during the past decade. However, the lack of analytical standards, and especially the time required for them to become available for newly identified modified mycotoxins, remains an important bottleneck in the effort to expand our understanding over a broader assortment of these substances. Future investigations focusing on the presence of modified mycotoxins in animal-derived products (milk, meat, eggs, etc.) could be of value to assess the potential carry-over along the food chain.

2.6 Metabolomics in mycotoxin research

"Metabolomics" was defined in the 1990s as the comprehensive analysis of the whole metabolome of an organism, which represents the entire complement of its metabolites (Oliver et al., 1998), and is one of the three keystone functional genomics technologies alongside transcriptomics (i.e. gene expression changes at the RNA level) and proteomics (i.e. protein expression changes) (van der Werf and Mariët, 2005). The general application of metabolomics is to provide insight into complex biological processes and eventually understand the relationship between an organism's genotype and phenotype. In essence, metabolomics experiments aim to generate a snapshot of the metabolic state of an organism and characterise the fluctuations in the abundances of measured metabolites arising from natural processes or external, experimental biotic or abiotic perturbations (Schuhmacher et al., 2013). It is important to note that there is a clear distinction between primary metabolism, which refers to all essential anabolic and catabolic processes required for cell maintenance and proliferation, from secondary metabolism, which is not essential for the survival of cells and was described as early as 1891 by Kössel (Kliebenstein, 2004). Secondary metabolites are a very diverse group of compounds and their production is highly inducible, meaning that their abundance is associated with complex environmental, developmental and physiological factors.

The utilisation of metabolomics in the study of plants, diseases and the environment is steadily increasing. The field has markedly advanced during the previous couple of decades owing to important developments in analytical methodologies and data processing software and has found a prominent role in mycotoxin research. From an analytical perspective, metabolomics constitutes a huge challenge, as it deals with the determination of largely chemically heterogeneous mixtures of metabolites with concentrations that can range from pico- to milli-molar levels. One of the strategies applied in mycotoxin metabolomics studies is metabolite profiling, which is defined as the (semi)quantitative analysis of a set of metabolites of a sample (Kluger et al., 2015a). Most such experiments rely on LC–HRMS and NMR spectroscopy for metabolite profiling and fingerprinting; the two analytical techniques can also be regarded as complimentary to each other. NMR methods can deal with complex sample matrices without the need for sample preparation and produce signals that corelate directly and linearly with compound abundance, although lacking high sensitivity (Lu et al., 2008). On the other hand, LC–HRMS offers high selectivity and sensitivity and is becoming an integral part to most metabolomics approaches.

Analytical methods have primarily been developed and utilised for routine measurements of the 400 or so currently known mycotoxins. However, extreme weather conditions that cause rapid changes in fungal biodiversity may affect mycotoxins in food and feed (Paterson and Lima, 2010). Moreover, living organisms (plants, mammals and

microorganisms), as well as food manufacturing processes can chemically alter the structure of mycotoxins and as a result tremendously expand the body of compounds potentially relevant to safety (Berthiller et al., 2013). In contrast to the plethora of chemical knowledge that is available for parent mycotoxins, very little is currently known about the chemistry of modified mycotoxins. In this context, metabolomics approaches have been increasingly gaining momentum over the past decade in mycotoxin-related studies and becoming the cornerstone in metabolite profiling for understanding plant resistance mechanisms and plant–pathogen interactions (Righetti et al., 2016).

2.6.1 Targeted and untargeted metabolomics

The two fundamental concepts in metabolomics are targeted and untargeted methodologies. Targeted approaches are generally hypothesis-driven and aim at the detection of chemically defined metabolites, focusing on one or more metabolic pathways (Patti et al., 2012). In targeted approaches, analytical procedures are commonly tailored to accommodate the physicochemical properties of the specified set of analytes of interest, for which chemical identification and absolute quantification are usually possible. Targeted analyses are mostly performed with TQ–MS with SRM, allowing for highly sensitive, robust and relatively high-throughput measurements of a wide range of small molecules. There is a wealth of published literature describing optimal protocols for sample preparation and targeted analysis of mycotoxins and derivatives in a variety of biological matrices (Berthiller et al., 2017; Berthiller et al., 2018; Tittlemier et al., 2019).

On the other hand, untargeted metabolomics are global in scope and strive to simultaneously measure as many metabolites as possible, including those currently unknown at the time of measurement. In a broad sense, untargeted metabolomics could also be described as screening against a large database of compounds or a retrospective analysis of compounds not specifically anticipated (Righetti et al., 2016). LC–HRMS (e.g. QTOF, Orbitrap) is the most commonly utilised analytical tool in untargeted metabolomics research. However, as no reference standards are available for most newly detected metabolites, only relative concentrations can be determined and not exact levels.

2.6.2 Stable isotopic labelling-assisted untargeted metabolomics

Analysis of biological samples with LC–HRMS instrumentation can result in the detection of several hundreds to thousands of peaks from a single analysis. Each of these peaks is referred to as a "feature" and corresponds to a detected ion signal with a unique *m/z* and retention time that is constant throughout a set of biological samples, whereas the intensity may significantly vary between samples (Kuhl et al., 2011). It is worth noting that eluting metabolites may give rise to numerous ion species and thus produce more than one feature, including multiply charged ions, several adduct ions (e.g. sodium, ammonium, potassium), in-source fragments and isotopologues carrying a varying number of heavier isotopes (e.g. ^{13}C, ^{15}N) (Bueschl et al., 2014). All features belonging to the same metabolite are termed a "feature group" and share the same chromatographic retention time and peak shape. For automated data processing of LC–HRMS-derived metabolomics datasets, several software packages and algorithms, such as MetAlign, Maven and XCMS, to name a few, have been developed and are frequently used in the field of metabolomics (Kluger et al., 2015a).

In untargeted metabolomics, effective data processing, reliable annotation/identification and accurate quantification of metabolites constitute major challenges (Scalbert et al., 2009; Patti et al., 2012; Dunn et al., 2013). However, metabolomic profiling using stable isotopic labelling (SIL)-assisted approaches offers great potential to tackle these challenges and allow in-depth investigations of the metabolic fate of certain tracer compounds. SIL metabolomic profiling is an innovative strategy to monitor and interpret the resulting metabolome, following the interactions of biological systems with artificially labelled molecules, employing stable isotopes of elements (e.g. ^{13}C- or ^{15}N-labelling). Compared to non-labelled pendants (compounds with a natural isotopic distribution), labelled molecules have a higher molecular weight due to their enrichment with low abundance naturally occurring stable isotopes. As native and labelled compounds possess identical physico-chemical properties, they perfectly co-elute with very similar chromatographic peak shapes and can be easily separated by their *m/z* and show distinct isotope patterns in HRMS data (Bueschl et al., 2014). By monitoring pairs of non-labelled and labelled precursor molecules, a holistic view of the metabolism of any given tracer can be achieved. MetExtract, the data processing software utilised in Studies III and IV, is a computational tool aiming at the untargeted automated global detection of LC–HRMS signals originating from metabolites generated within a biological system (Bueschl et al., 2012). Thus far, only a handful of publications in the literature have employed SIL-assisted untargeted metabolomics for mycotoxin research. In the first ever application of this workflow in mycotoxin research, (Kluger et al., 2015b) investigated the metabolism of DON in wheat

and reported a total of nine DON biotransformation products. A very similar experimental setup was used by Meng-Reiterer et al. (2016), to unravel the metabolism of HT2 and T2 in oats. The authors annotated 16 HT2 and 17 T2 metabolites, including novel glucosylated and hydroxylated forms. The SIL-assisted untargeted approach was also very recently used to study the fate of DON and identify its degradation products during the baking of crackers, biscuits and bread (Stadler et al., 2019).

It worth mentioning that to unequivocally identify a metabolite, its retention time and MS/MS spectrum need to be compared to an authentic reference standard. For the majority of compounds detected in untargeted metabolomics, however, no reference standards are available and only putative annotation can be achieved, even with the most advanced MS instrumentation. LC–HRMS analysis does not provide information on the exact structure and stereochemistry of the analytes, and the reported structures can consequently only be regarded as putative until characterised by NMR spectroscopy.

2.7 Risk assessments and regulations

The knowledge that mycotoxins can have serious health effects on consumers and livestock has led more than 100 countries to establish regulations and specific limits for a number of these compounds in food and feed (FAO, 2004). Current regulations are based on scientific opinions issued by international organisations such as JECFA and EFSA, the latter having undertaken the responsibilities previously handled by the Scientific Committee on Food (SCF) in the EU. The establishment of legislative limits (i.e. maximum/indicative levels or guidance values) of acceptability of certain mycotoxins in food- and feedstuffs, as well as law-making are enabled by surveillance projects on worldwide mycotoxin contamination, risk assessments and consideration of socio-economic factors (van Egmond et al., 2007). Risks associated with mycotoxins are dependent on the intrinsic hazardous properties of the toxin and expected exposure.

JECFA, SCF and EFSA have published scientific opinions and/or established guidance values for all the precursor mycotoxins examined in this work. Between the years 2000 and 2001, JECFA conducted safety evaluations of several mycotoxins and established PMTDI values for ZEN (0.5 µg/kg bw), DON (1 µg/kg bw) and the sum of HT2 and T2 (0.06 µg/kg bw). Around the same period, SCF published a series of opinions for DON, NIV and the sum of HT2 and T2 (summarised in SCF, 2002), as well as for ZEN (SCF, 2000). In these opinions, the following tolerable daily intake (TDI) values were established: a TDI of 1 µg/kg bw for DON; a temporary TDI of 0.2 µg/kg bw for ZEN; a temporary TDI of 0.7 µg/kg bw for NIV; and a combined temporary TDI for HT2 and T2 of 0.06 µg/kg bw.

Following the work of SCF, EFSA maintained the TDI value for human exposure of 1 µg/kg bw for DON without considering, at the time, specific measures for the

metabolites 3Ac- and 15Ac-DON. On the other hand, the TDI of ZEN was lowered to 0.25 μg/kg bw, after applying the necessary uncertainty factors, based on a no observed effect level (NOEL) of 10 μg/kg bw per day for oestrogenic effects in piglets (EFSA, 2011). In the same year, EFSA adopted a scientific opinion with respect to the risks incurred by the presence of HT2 and T2, concluding with a combined TDI of 0.1 μg/kg bw due to immunological effects in pigs. In the opinion, it was argued that both mycotoxins would be included under a common TDI because of the rapid metabolism of T2 to HT2 (EFSA, 2011). The CONTAM Panel also derived a TDI for NIV (1.2 μg/kg bw), from a 90-day rat study in which reduced white blood cell counts were observed (EFSA, 2013).

In the EU, risk assessments of mycotoxins have resulted in regulations that aim to protect consumers and livestock from the harmful effects of certain mycotoxins. Specifically, Commission Regulation (EC) No 1881/2006 (EC, 2006b) and its amendments have established maximum levels (MLs) for aflatoxin B_1, aflatoxin M_1, the sum of aflatoxins B_1, B_2, G_1 and G_2, ochratoxin A, patulin, DON, ZEN and the sum of fumonisins B_1 and B_2 in relevant food categories. Consequently, foodstuffs listed therein that contain any of these mycotoxins, at a concentration exceeding the MLs set out in the regulation, must not be placed on the EU market. For the type A trichothecenes HT2 and T2, Commission Recommendation 2013/165/EU (EC, 2013) has been published, providing indicative values for the sum of the two toxins in cereals, cereal-derived products and (compound) feed. For the presence of other mycotoxins in feed, MLs have only been set for aflatoxin B_1 and rye ergot (*Claviceps purpurea*) in Directive 2002/32/EC (EC, 2002b); guidance values have been set for the presence of DON, ZEN, ochratoxin A and the sum of fumonisins B_1 and B_2 in products intended for animal feeding (Commission Recommendation 2006/576/EC; EC, 2006c). Currently, no legal MLs exist in the EU for NIV in food or feed products (EFSA, 2013). At the time of writing, no legislative limits for modified forms of *Fusarium* mycotoxins exist, either. Table 2 presents the MLs, indicative levels and guidance values of the native *Fusarium* mycotoxins studied in this thesis, in different types of commodities derived from small grain cereal grains.

Regulators are nowadays reassessing the risk of *Fusarium* mycotoxins by taking into account the presence of modified mycotoxins in food and feed. At first, JECFA proposed to include to the existing PMTDI of 1 μg/kg bw for DON, its acetylated congeners 3Ac-DON and 15Ac-DON (JECFA 2011). In this regard, the Committee considered the toxicities of 3Ac-DON and 15Ac-DON as equal to that of DON and thus as contributing factors to the total DON-induced toxicity. However, at that time, DON3Glc was not included in the group PMTDI due to insufficient toxicological information. JECFA experts also noted that absorption, distribution, metabolism and excretion (ADME) studies and occurrence survey reports were needed for this compound.

Table 2. Maximum levels, indicative levels and guidance values for the *Fusarium* mycotoxins DON, HT2, T2 and ZEN in food and feed, according to Commission Regulation (EC) No 1881/2006 with amendments, Commission Recommendation 2006/576/EC and Commission Recommendation 2013/165/EU.

Food/feed matrices	**Mycotoxin(s)**		
	DON	**Sum of HT2 and T2**	**ZEN**
Feed and compound feed [α]	900–12,000 µg/kg	250–2000 µg/kg	100–3000 µg/kg
Unprocessed cereals [β]	1250–1750 µg/kg	100–1000 µg/kg	100–350 µg/kg
Milling fractions and products [β]	750–1250 µg/kg	50 µg/kg	200–300 µg/kg
Cereals intended for direct human consumption, flour, pasta, bread, snacks, etc. [β]	500–750 µg/kg	25–200 µg/kg	50–400 µg/kg
Processed cereal-based foods and baby foods [β]	200 µg/kg	15 µg/kg	20 µg/kg

[α] Guidance values relative to a feedstuff with a moisture content of 12%, except for HT2 and T2, which are indicative values.
[β] Maximum levels, except for HT2 and T2, which are indicative values.

Recently, a succession of scientific opinions published by the CONTAM Panel of EFSA have highlighted the risks of modified mycotoxins for human and animal health and provided updated guidance values; the opinions are presented here in chronological order. In the case of ZEN, the panel decided to set a group-TDI of 0.25 µg/kg bw expressed as ZEN equivalents for ZEN and its modified phase I and II metabolites (EFSA, 2016). Later, in 2016, another EFSA opinion was published establishing guidance values for HT2, T2 and their modified forms. A group-TDI of 0.02 µg/kg bw and a group-acute reference dose (ARfD) of 0.3 µg/kg bw were derived for the sum of HT2, T2 and their phase I and II metabolites. Relative potency factors were assigned to conjugated metabolites, which are not toxic *per se*, but could, given suitable conditions, release their toxic aglycones. For NIV, no new relevant data have become available since the publication of the previous opinion, and the TDI of 1.2 µg/kg bw and the ARfD of 14 µg/kg bw thus remained unchanged in EFSA's latest evaluation of NIV (EFSA, 2017c). However, NIV3Glc was included in these values because of its presumed potential to hydrolyse back to its precursor mycotoxin. Lastly, for DON, EFSA maintained the same value of 1 µg/kg bw but transformed it into a group-TDI to include the modified derivatives 3Ac-DON, 15Ac-DON and DON3Glc (EFSA, 2017); a group-ARfD of 8 µg/kg bw per eating occasion was also calculated.

3 AIMS

The rationale behind this Ph.D. thesis was to study the formation and natural occurrence of modified *Fusarium* mycotoxins in cereals, as well as their metabolic fate in beer fermentation. More specifically, the aims of the thesis were:

- To develop rapid, accurate and reliable analytical methods based on LC–MS/MS for the simultaneous determination of certain *Fusarium* mycotoxins and their modified forms in cereal grains (I and II);

- To conduct a nationwide survey in Finnish cereal grains for the natural occurrence of *Fusarium* mycotoxins and their modified forms (II);

- To unravel the metabolism and identify potential biotransformation products of the *Fusarium* type A trichothecenes HT2 and T2 in barley and wheat, via the utilisation of state-of-the-art analytical methodology and SIL-assisted untargeted metabolomics (III and IV);

- To investigate the metabolic fate of DON, DON3Glc, HT2 and T2 during beer fermentation and their potential to cause adverse effects in brewing yeast (V).

4 MATERIALS AND METHODS

4.1 Experimental overview

The experimental work of this Ph.D. dissertation consists of five research studies (I–V). Study I was carried out in the Chemistry Unit of the Finnish Food Authority (Ruokavirasto), Helsinki, Finland (previously: Chemistry and Toxicology Unit, Finnish Food Safety Authority, Evira) and in the Special Solutions Center of Thermo Fisher Scientific (Dreieich, Germany). The experimental work of Study II was conducted in the laboratory facilities of the Finnish Food Authority in collaboration with the University of Natural Resources and Life Sciences, Vienna (BOKU), Austria. The *in planta* metabolomics Studies III and IV were performed in the Institute of Bioanalytics and Agro-Metabolomics and the Institute for Biotechnology in Plant Production of the Department of Agrobiotechnology, IFA-Tulln (BOKU). For Study V, the brewery fermentation and the analysis of yeast and spent medium were carried out in the VTT Technical Research Centre of Finland (Espoo, Finland). For the same study, mycotoxin determination and identification of resulting metabolic products in wort and yeast samples were performed in the Finnish Food Authority, with additional measurements taking place at the Department of Food and Environmental Sciences of the University of Helsinki (Helsinki, Finland).

In Study I, the performance of on-line clean-up in comparison to conventional mycotoxin sample preparation techniques for LC–MS/MS determination of DON and its modified form, DON3Glc, in barley and wheat was assessed. Study II describes the development of a multi-analyte LC–MS/MS method for the simultaneous determination of *Fusarium* mycotoxins and several of their currently known modified metabolites. The method was in-house validated and utilised for the analysis of naturally contaminated Finnish cereal grains. In Studies III and IV, untargeted metabolic profiling approaches based on SIL and LC–HRMS/MS were applied in order to investigate the metabolism of HT2 and T2 in both barley and wheat. Finally, Study V was performed to examine the metabolic fate of DON, DON3Glc, HT2 and T2, as well as their potential adverse effects on lager yeast during brewery fermentation.

4.2 Chemicals and reagents

Acetic acid, acetonitrile, ammonium acetate, ammonium formate, ammonium hydroxide (25%), formic acid, hexane and methanol, all of HPLC grade or higher, were purchased

from one or more of the following vendors: Agilent Technologies (Waldbronn, Germany), J.T. Baker (Deventer, the Netherlands), Sigma-Aldrich (Steinheim, Germany and Vienna, Austria), Thermo Fisher Scientific (Langenselbold, Germany) and VWR (Leuven, Belgium and Vienna, Austria). Tween 20 was obtained from Sigma-Aldrich (Vienna, Austria) and Celite 545 from Merck (Billerica, MA, USA). Water was purified by reverse osmosis with a Millipore Milli-Q Plus system (Espoo, Finland) in Studies I, II and V, and with an ELGA Purelab Ultra-AN-MK2 system from Veolia (Vienna, Austria) in Studies III and IV.

Analytical standards of HT2, T2, 3Ac-DON, DON, NIV, ZEN, α-ZEL, β-ZEL in solid form and DON3Glc (50 µg/mL in acetonitrile) were purchased from Sigma-Aldrich (Steinheim, German). Additional HT2 and T2 standards that were used in Study V were obtained from Romer Labs (Tulln, Austria). Crystalline non-labelled HT2 (purity 92%) and T2 (purity 85%), as well as uniformly labelled U-[$^{13}C_{22}$] HT2 (purity 86%; degree of enrichment 99.3 atom % ^{13}C) and U-[$^{13}C_{24}$] T2 (purity 98%; degree of enrichment 99.6 atom % ^{13}C) of Studies III and IV were purchased from Romer Labs GmbH (Tulln, Austria). The modified mycotoxins DON3Glc (Berthiller et al., 2005), ZEN14Glc, α-ZEL14Glc, β-ZEL14Glc (Berthiller et al., 2009), ZEN16Glc (Kovalsky Paris et al., 2014), ZEN14Sulf (Mikula et al., 2013), HT2-3-Glc and NIV3Glc (Michlmayr et al., 2018), used in Studies II–IV, were enzymatically produced or chemically synthesised by BOKU. Finally, 3-acetyl-T2 toxin (3Ac-T2) was synthesised from T2 using acetic anhydride in dichloromethane/pyridine with 4-dimethylaminopyridine as catalyst. The chemical structures and purities (≥ 95%) of all resulting modified mycotoxins were verified by NMR and LC-UV measurements. The standard T-2 toxin-α-glucoside (T2-α-Glc) was prepared as described in McCormick et al. (2015).

4.3 Samples and spiking experiments

4.3.1 Finnish cereal grains

Cereal grains analysed for *Fusarium* mycotoxins and their modified forms in the survey study (II) were randomly obtained from the quality monitoring programme of the Finnish grain harvest. The quality monitoring programme is based on samples sent in by farmers. Each sample weighed 2 kg and consisted of several subsamples collected from arbitrary points of a particular field. In total, 95 samples were analysed, consisting of 34 barley (50% malting varieties, 38% feed varieties and 12% for other purposes), 31 oat (16% food varieties, 84% feed, farm trade and seed varieties) and 30 spring wheat (77% food varieties, 13% feed varieties and 10% seed varieties), all samples from the 2013 harvest (n = 1117). These samples represented commonly cultivated small grain cereal varieties

in Finland from different geographic areas (Figure 6). The 2013 Finnish grain harvest quality monitoring programme targeted mainly high-risk areas regarding mycotoxin contamination.

Area	Cereal grains (n = 95)		
	Barley	Oats	Wheat
1	4	3	6
2	10	1	5
3	2	1	5
4	5	3	3
5	2	1	3
6	0	2	4
7	2	2	0
8	0	3	1
9	1	2	0
10	0	3	0
11	5	6	2
12	3	1	0
13	0	3	1
14	0	0	0
15	0	0	0
Total:	34	31	30

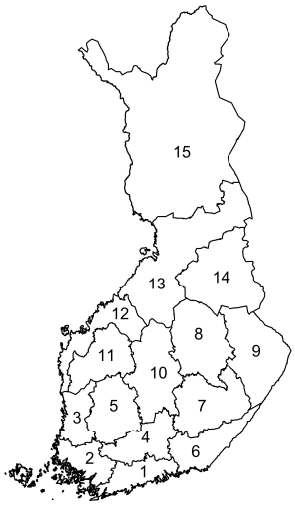


Figure 6. Origin and number of the Finnish cereal grains analysed in Study II.

4.3.2 Plant materials and mycotoxin treatment

Barley (*Hordeum vulgare* L. sensu lato) of the variety "Calcule" was selected for the qualitative screening and time-course experiment in Study III. For the metabolomics profiling experiment (IV), the awn-free spring wheat (*Triticum aestivum* L.) variety "Remus" ("Sappo"/"Mex"/"Famos") was used, which is highly susceptible to FHB. The time-course experiment of Study IV was conducted on both Remus and the FHB-resistant variety "CM-82036-1TP-10Y-OST-10Y-OM-OFC" (abbreviated to "CM-82036"), which possess awns and originates from the cross of the varieties "Sumai-3" and "Thornbird".

Initially, barley and wheat seeds were germinated and planted into pots. The pots (23 cm diameter) were filled with seven-litre portions of a substrate, prepared from of a mixture of 500 L heat-sterilised compost, 250 L peat, 10 kg sand and 250 g rock flour. Five seedings of each plant variety were planted in each pot. During the experiments, pots were watered typically three times per week. Plants used for qualitative screening were grown in the greenhouse and after tillering were transferred to a growth chamber with computer-controlled settings for light, temperature and relative humidity. Light intensity was 560 µmol/sm^2 for barley and 360 µmol/sm^2 for wheat at 1 m above the soil. Relative air humidity was set between 60% and 70% during

plant growth. Temperature (day/night) and duration of illumination (hours) varied according to the developmental stage of the plants: after planting until the end of tillering, 12 °C/10 °C/12 h; end of tillering to mid-stem extension when the ear starts to swell, 14 °C/10 °C/14 h; mid-stem extension to start of heading, 16 °C/14 °C/14 h; from the start of heading until the start of flowering, 18 °C/14 °C/14 h; and from the start of flowering until the end of the experiments, including application of the test solutions and sampling, 20 °C/18 °C/16 h.

The treatment of barley and wheat plants commenced at the flowering stage with three biological replicates per treatment group (HT2, T2 and mock). Toxin/mock solutions were applied to only one ear of each plant to prevent potential systemic effects related to previously treated ears. For the qualitative screening experiments, injection of the respective test solutions (5 µL per spikelet in barley and 10 µL per spikelet in wheat) was performed with an electronic pipette into each outer floret of a spikelet. In the metabolic profiling experiments, a 50:50 (v/v) mixture of ^{13}C-labelled and non-labelled test solutions of HT2 or T2 were applied; mock ears were treated only the with spiking solution. In total, 180 and 200 µg of the ^{12}C/^{13}C toxin mixtures were applied in adjacent barley and wheat spikelets, respectively, at time points 0, 48, 96, 120 and 144 h. Sampling was performed 24 h after the last spiking.

For the time-course experiments, ears were treated similarly except that a single dose of 200 µg non-labelled toxin was injected in 10 pairs of neighbouring spikelets on one ear. Samples were collected at 0, (6), (12), 24, (48) and 72 h (time points in parenthesis collected only from wheat treated samples), as well as after one week and at full ripening. Barley and wheat ears were weighed, flash-frozen in liquid nitrogen and stored at −80 °C until analysis. Only the middle (treated) parts of ears were analysed.

4.3.3 Brewing fermentation experiments

The fermentation experiments of Study V were performed at the 100-mL scale with the lager yeast *S. pastorianus* VTT A-63015 (abbreviated as A15), which was obtained from the VTT Culture Collection (http://culturecollection.vtt.fi). Erlenmeyer flasks contained 97.5 g of 11.5° Plato unhopped wort and 2.5 g of yeast suspension, giving a pitching rate of 5 g/L. Wort contained DON, HT2 and T2 individually at either low (100 µg/L) or high (10,000 µg/L) concentrations. Another batch was prepared with 400 µg/L DON3Glc or a mixture of T2 and HT2 (5000 µg/L each). Control wort contained 200 µL acetonitrile and all dose groups were prepared in triplicate. Cultivations lasted for four days and were carried out at 15 °C under aerobic conditions and with shaking (120 rpm). A 5-mL sample was taken directly before and directly after inoculation. Two samples of 5 mL were taken daily in the first two days and once daily thereafter, resulting in the following

time points: 0, 4, 24, 28, 48, 72 and 96 h. Samples were centrifuged and the supernatant was flash-frozen in liquid nitrogen and stored at −80 °C until analysis.

4.3.4 Method validation samples

In Study I, commercially available finely ground barley and wheat flours (Myllyn Paras Oy, Hyvinkää, Finland) were used as blank material, after ensuring no/minimal contamination of target analytes. These barley and wheat flours (20 ± 0.01 g) were spiked with either 200 µg/kg DON and 50 µg/kg DON3Glc (low concentration level) or 750 DON µg/kg and 160 µg/kg DON3Glc (high concentration level), with six replicates for each level. For Study II, the same types of barley and wheat flours were used for validation. For oat matrix validation, oat kernels from the Finnish grain quality monitoring programme were used that were finely ground and analysed for the absence of target analytes prior to spiking. Three spiking levels, with six replicates, were prepared for each matrix with varying concentrations of the 16 target analytes included in the method: 3Ac-DON, DON, DON3Glc, HT2, HT2-3-Glc, NIV, NIV3Glc, T2, ZEN, ZEN14Glc, ZEN14Sulf, ZEN16Glc, α-ZEL, α-ZEL14Glc, β-ZEL and β-ZEL14Glc. Concentration levels ranging between 5 and 800 µg/kg were added into 0.500 ± 0.005 g of cereal flour and were allowed to soak overnight in the dark at room temperature. The process was repeated on three days. Barley (variety Calcule) and wheat (variety Remus) samples of the mock groups were used for method validation in Studies III–IV. An acetonitrile solution containing HT2, T2, HT2-3-Glc and 3Ac-T2 (and T2-α-Glc only for barley) was spiked into 100 ± 2 mg of blank sample at one concentration of 1500 µg/kg in biological triplicate and stored overnight at room temperature. Lastly, for the in-house validation of Study V, blank wort was spiked with either 80 or 8000 µg/L DON, HT2 and T2 or a single concentration of 250 µg/kg DON3Glc, all in three replicates. Non-spiked blank samples were also analysed in all studies as part of method validation.

4.4 Sample preparation

The samples prepared in Study I were first extracted with 80 mL acetonitrile:water (84:16, v/v) by mixing in a horizontal shaker for 2 h, and afterwards 8 mL was used for each of the following sample preparation techniques: i) extract and shoot, ii) purification with MycoSep 227 clean-up columns (Romer Labs Inc., Union, MO, USA), iii) MycoSep 227 with an additional 8 mL acetonitrile elution step, iv) centrifugal filtration with Amicon® Ultra-15 filter devices (Millipore, Schwalbach, Germany) and v) automated on-line sample clean-up with the TLX–1 system (Thermo Fisher Scientific, Franklin, MA, USA) utilising TurboFlow™ chromatography. It is worth noting that the selection of

conventional sample preparation approaches was by no means exhaustive, as immunoaffinity columns, QuEChERS and several other commercially available techniques were not assessed in this study.

Barley, oat and wheat samples analysed during the survey study (II) were finely ground and extracted using 2 mL of acetonitrile:water:acetic acid (79:20:1, v/v/v) per 0.500 ± 0.005 g sample. After the 90-min extraction, 350 µL of the supernatant was transferred into HPLC vials and diluted with an equal amount of acetonitrile:water:acetic acid (20:79:1, v/v/v) before analysis with LC–MS/MS. For oats, an additional step to remove fat with the addition of 1 mL hexane to the extract (ca. 1.2 mL) was implemented after extraction. The mixture was shaken for 10 min and centrifuged at 2700 × g for 5 min. The hexane layer was then removed, and 350 µL of the extract layer was diluted with an equal amount of acetonitrile:water:acetic acid (20:79:1, v/v/v) and transferred into HPLC vials for analysis.

The same extract and shoot based sample preparation process was used in both Studies III and IV. Frozen barley and wheat ears were ground into a fine powder with a ball mill for 30 s at 30 Hz under cooled conditions (liquid nitrogen). After homogenisation, 100 ± 2 mg of sample was extracted with 500 µL of acetonitrile:water:formic acid (79:20.9:0.1, v/v/v) for 90 min. After centrifugation at 22,750 × g, supernatants (ca. 200 µL) were transferred into HPLC vials and diluted depending on whether the samples were prepared for quantification or metabolite annotation/identification with analytical instrumentation outlined in Table 3.

In Study V, 2 mL of wort was transferred into test tubes with 7.9 mL acetonitrile, 0.1 mL acetic acid and 0.25 g Celite. After 1 h of shaking at 200 rpm, the extracts were filtered through folded paper filters. Aliquots of 3.5 mL were taken in duplicate from each sample and evaporated to dryness at 40 °C. Samples were reconstituted with 500 µL of acetonitrile:water (50:50, v/v) and transferred into HPLC vials for analysis. For yeast analysis, a 20% (w/v) yeast suspension was prepared by adding 4 mL water for each 1 g of fresh yeast mass. A 50-µL sample was added to 250 µL of extraction solvent consisting of acetonitrile:methanol (2:1, v/v) and homogenised in a ball mill. After homogenisation, the samples were centrifuged to remove solid particles and stored at −80 °C until analysis.

4.5 Analytical methods

Chromatographic and MS parameters of all methods developed and used for the purposes of the experiments of this thesis are described in detail in papers I–V. Table 3 presents an overview of these methods.

Table 3. Overview of the analytical methods used in Studies I–V for the determination of *Fusarium* mycotoxins and their modified forms.

Analytes	Matrices	Sample preparation [a]	Mobile phase [a] (gradient elution)	Stationary phase	Apparatus	Mass range	Study
DON, DON3Glc	barley, wheat	Extraction: 20 g sample + 80 mL ACN:H_2O (84:16, v/v) Clean-up: None (extract and shoot), MycoSep® 227; Romer Labs or Amicon® Ultra-15 centrifugal filter devices; Millipore	A: ACN:H_2O (90:10 v/v) with 0.1% NH_4OH (v/v) B: ACN:H_2O (30:70 v/v) with 0.1% NH_4OH (v/v)	ACQUITY UPLC BEH Amide column (100 x 2.1 mm, 1.7 µm) with BEH Amide VanGuard pre-column; Waters	ACQUITY UPLC® and Xevo™ ESI–TQ–MS; Waters	SRM	I
DON, DON3Glc	barley, wheat	Extraction: 20 g sample + 80 mL ACN:H_2O (84:16, v/v) Clean-up: Transcend™ TLX-1 with TurboFlow™ MCX-2 column (50 x 0.5 mm); Thermo Scientific	A: H_2O with 0.1% HCOOH (v/v) B: MeOH with 0.1% HCOOH (v/v)	Hypersil Gold C18 column (50 x 4.6 mm, 5 µm); Thermo Scientific	CTX AS™ with two Accela 1250 Pumps™ and Exactive™ Orbitrap ESI–MS; Thermo Scientific	*m/z* 200–500 full scan mode and *m/z* 100–300 with HCD fragmentation	I
HT2, T2, DON, NIV, ZEN, 3Ac-DON, α-ZEL, β-ZEL, DON3Glc, HT2-3-Glc, NIV3Glc, ZEN14Glc, ZEN14Sulf, ZEN16Glc, α-ZEL14Glc, β-ZEL14Glc	barley, oats, wheat	Extraction: 0.5 g sample + 2 mL ACN:H_2O:CH_3COOH (79:20:1, v/v/v) Clean-up: None for barley and wheat (extract and shoot). For oats, defatting with 1 mL n-C_6H_{14}	A: H_2O with 10 mM $NH_4CH_3CO_2$ B: ACN	Atlantis® T3 column (150 x 3.0 mm, 3.0 µm) with T3 pre-column; Waters	ACQUITY UPLC® and Xevo™ ESI–TQ–MS; Waters	SRM	II
HT2, T2, 3Ac-T2, HT2-3-Glc, T2-α-Glc and metabolites	barley	Extraction: 100 mg + 500 µL ACN:H_2O:HCOOH (79:20.9:0.1, v/v/v) Clean-up: None (extract and shoot)	A: H_2O with 0.1% HCOOH (v/v) and 5 mM NH_4HCO_2 B: MeOH with 0.1% HCOOH (v/v) and 5 mM NH_4HCO_2	ZORBAX SB-C18 column (150 x 2.1 mm, 3.5 µm); Agilent Technologies	UltiMate® 3000 HPLC and Exactive™ Plus Orbitrap ESI–MS; Thermo Fisher Scientific	*m/z* 130–1300 full scan mode	III

Table 3. Continued.

HT2, T2, 3Ac-T2, HT2-3-Glc, T2-α-Glc and metabolites	barley	Extraction: 100 mg + 500 µL ACN:H_2O:HCOOH (79:20.9:0.1, v/v/v) Clean-up: None (extract and shoot)	A: H_2O with 0.1% HCOOH (v/v) and 5 mM NH_4HCO_2 B: MeOH with 0.1% HCOOH (v/v) and 5 mM NH_4HCO_2	ZORBAX SB-C18 Rapid Resolution HD column (150 x 2.1 mm, 1.8 µm); Agilent Technologies	1290 Infinity UHPLC and 6550 iFunnel ESI–QTOF–MS; Agilent Technologies	*m/z* 50–1500 full scan mode	III
HT2, T2, HT2-3-Glc, 3Ac-T2 and metabolites	wheat	Extraction: 100 mg + 500 µL ACN:H_2O:HCOOH (79:20.9:0.1, v/v/v) Clean-up: None (extract and shoot)	A: H_2O with 0.1% HCOOH (v/v) (and 5 mM NH_4HCO_2) B: MeOH with 0.1% HCOOH (v/v) (and 5 mM NH_4HCO_2)	ZORBAX SB-C18 Rapid Resolution HD column (150 x 2.1 mm, 1.8 µm); Agilent Technologies	1290 Infinity UHPLC and 6550 iFunnel ESI–QTOF–MS; Agilent Technologies	*m/z* 50–1500 full scan mode	IV
HT2, T2, DON, 3Ac-DON, DON3Glc and metabolites	wort, yeast	Extraction (wort): 2 mL wort + 7.9 mL ACN + 0.1 mL CH_3COOH + 0.25 g Celite Extraction (yeast): 20% (w/v) yeast suspension (4 mL H_2O/1 g fresh yeast mass). 50 µL yeast suspension + 250 µL ACN:MeOH (2:1, v/v) Clean-up: None (extract and shoot)	A: H_2O with 10 mM $NH_4CH_3CO_2$ B: ACN	Atlantis® T3 column (150 x 3.0 mm, 3.0 µm) with T3 pre-column; Waters	ACQUITY UPLC® and Xevo™ ESI–TQ–MS; Waters	SRM	V
HT2, T2, DON, 3Ac-DON, DON3Glc and metabolites	wort	Extraction: 2 mL wort + 7.9 mL ACN + 0.1 mL CH_3COOH + 0.25 g Celite	A: H_2O with 10 mM $NH_4CH_3CO_2$ B: ACN	Atlantis® T3 column (150 x 3.0 mm, 3.0 µm) with T3 pre-column; Waters	ACQUITY UPLC® and Micromass Premier™ ESI–QTOF–MS; Waters	*m/z* 100–1000 full scan mode	V
HT2, T2, DON, 3Ac-DON, DON3Glc and metabolites	wort	Extraction: 2 mL wort + 7.9 mL ACN + 0.1 mL CH_3COOH + 0.25 g Celite	A: H_2O with 10 mM $NH_4CH_3CO_2$ B: ACN	Atlantis® T3 column (150 x 3.0 mm, 3.0 µm) with T3 pre-column; Waters	ACQUITY UPLC® and SYNAPT G2-Si ESI–QTOF–MS; Waters	*m/z* 50–1500 full scan mode	V

[α] ACN, acetonitrile; H_2O, water; MeOH, methanol; CH_3COOH, acetic acid; n-C_6H_{14}, n-hexane; HCOOH, formic acid; NH_4OH, ammonium hydroxide; $NH_4CH_3CO_2$, ammonium acetate; NH_4HCO_2, ammonium formate.

4.6 Method validation

All analytical methods used in Studies I–V were in-house validated by spiking experiments in cereal matrices, according to legislative criteria laid down in Commission Regulation (EC) No 401/2006 (EC, 2006) and Commission Decision 2002/657/EC (EC, 2002). Validation was performed in order to assess method performance by ensuring absolute quantification of target analytes, for which analytical standards were readily available for given matrices. Method validation parameters included specificity, linearity, apparent recovery (R_A), RSD_r, RSD_R, LOD and LOQ. Detailed information concerning the calculation of validation parameters is presented in the Materials and Methods section of each paper. In Studies II–V, matrix effects were also estimated and expressed as SSE, according to (Sulyok et al., 2006). Finally, extraction recovery (R_E) was determined as the ratio of R_A to SSE in Studies III and IV.

4.7 Data processing

4.7.1 Statistical data analysis

Validation data from Studies I–V were processed with Microsoft Excel® 2010 (Microsoft Co., Redmond, WA, USA). Statistical analysis in Study V was performed by one-way analysis of variance (ANOVA) and Tukey's HSD post hoc test ($p = 0.05$), using IBM SPSS software v. 22 (SPSS Inc., Chicago, IL, USA).

4.7.2 Untargeted metabolite screening

Acquired profile data from Studies III and IV were first centroided and converted to the mzXML data format (Pedrioli et al., 2004) with MSConvert from the ProteoWizard package (Kessner et al., 2008) version 3.0.3959 (http://proteowizard.sourceforge.net), before being processed with MetExtract software (Bueschl et al., 2012). This bioinformatics tool searches for characteristic isotope patterns of native (^{12}C or M and M+1) and partially ^{13}C-labelled (^{13}C or M' and M'–1) ion pairs.

The calculated *Δ m/z* difference between ^{12}C and ^{13}C ion pairs corresponds to n-labelled C-atoms, originating from the uniformly ^{13}C-labelled tracer: HT2 or T2 in these experiments. In samples treated with HT2, a mass difference of 22.0738 Da between the ^{12}C and ^{13}C ion pairs indicated the presence of the HT2 skeleton within a metabolite's molecular structure. Likewise, for metabolites containing an intact T2 backbone in their

structure, a difference of 24.0805 Da was measured between the ^{12}C and ^{13}C ion pairs. A lower ^{13}C count value from 22 and 24 for HT2 and T2, respectively, denoted metabolites that had undergone cleavage of one or more moieties originally present in their carbon skeleton. Figure 7 provides a schematic representation of the principle behind feature detection by MetExtract.

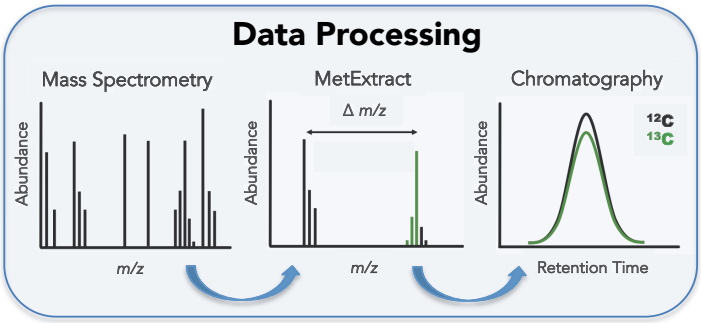


Figure 7. Schematic representation of feature detection using MetExtract software. Corresponding $^{12}C/^{13}C$ features are identified by a mass shift between monoisotopic and ^{13}C-labelled peaks of 22.0738 Da for HT2 and 24.0805 Da for T2, typical isotopic distribution patterns and a similar peak shape.

The thresholds set for a positive hit were that Δ *m/z* should have ≤ 4 ppm deviation from the theoretical value and that the observed $^{12}C/^{13}C$ abundance ratio between native and ^{13}C-labelled ion forms had to be 1.0 ± 0.5. EICs generated for M and M' ions were recognised as chromatographic peaks with the algorithm of (Du et al., 2006) and had to show a minimum Pearson correlation of 0.75. Such extracted ion pairs that were present in both EICs and had the same retention time (± 10 scans) were convoluted into feature groups (i.e. metabolites) using a minimum Pearson correlation of 0.85. Feature groups may have included quasi molecular ions, various adducts, in-source fragments and/or multiply charged ions; all derived from the same eluting metabolite.

4.7.3 Targeted metabolite screening

MetaboLynx™ XS software (Waters Co. Milford, MA, USA) was used for targeted metabolite screening of raw full scan LC–QTOF–MS data in Study V. MetaboLynx XS is a post-acquisition tool that compares MS data between control and treated samples and reveals peaks that could be attributed to biotransformation products by identifying variances in chromatographic retention times and signal intensities. After removing false positives, MS/MS experiments were conducted on those putative metabolites denoted by MetaboLynx XS and were characterised by manual spectral interpretation.

5 RESULTS

5.1 Conventional sample preparation *versus* on-line clean-up (I)

In Study I, an inter-laboratory method comparison was conducted to assess the performance of four conventional sample preparation techniques used in mycotoxin determination against automated on-line clean-up. Samples prepared with the conventional techniques, i)–iv), were analysed with a newly developed LC–ESI–TQ–MS/MS method, while samples processed with on-line clean-up, v), were analysed with an existing TLX–LC–HRMS method (Ates et al., 2013) after some necessary optimisation. Initially, the suitability of different columns was examined for both methods in terms of chromatographic separation and the retention of target analytes. For the LC–ESI–TQ–MS/MS method, a HILIC ACQUITY column (Waters) and an ACQUITY UPLC BEH amide column (Waters) were tested; for the TLX–LC–HRMS method, a reversed phase C 18, a polymeric Cyclone-P and an MCX 2 column were tested (all by Thermo Scientific). Sufficient retention, optimal peak shape and chromatographic separation of the target analytes DON and DON3Glc were achieved with the UPLC amide column using the former method and with the MCX 2 using the latter.

The methods were then in-house validated. Ion detection was performed in SRM mode with the TQ instrument, in which the parent ion of each analyte and two product ions were detected. For HRMS, identification of DON and DON3Glc was based on the presence of accurate mass ions of one parent ion and one fragment ion formed by higher-energy collision dissociation (HCD) fragmentation. Analyte retention times for both methods were in the range of ± 2.5%. The five-point calibration curves were linear over the working range of 100–1600 µg/kg and 20–320 µg/kg for DON and DON3Glc, respectively, with the coefficient of determination (R^2) values not being less than 0.985. Mean recoveries (%) ± %RSD of the spiking experiments are presented in Table 4. With the extract and shoot method, mean recoveries for DON and DON3Glc were between 94–99% (3–8%) in barley and 87–99% (1–10%) in wheat. MycoSep 227, ii) and iii), yielded acceptable recoveries for DON in barley, 74–94% (3–11%), and in wheat, 87–105% (4–8%), but not for DON3Glc, even with the additional acetonitrile elution step (recoveries < 30%). Application of centrifugal filtration resulted in recoveries within the acceptable range of 70–120%, but with slightly higher %RSD values than extract and shoot. Lastly, average recoveries (%RSD) with TurboFlow ranged between 73% and 96% (8–10%) in barley and 72% and 102% (5–12%) in wheat.

Table 4. Mean recoveries (%) of DON (200 and 750 µg/kg) and DON3Glc (50 and 160 µg/kg) from spiked barley and wheat samples with the following five sample preparation techniques: i) extract and shoot, ii) MycoSep® 227 clean-up column, iii) MycoSep 227 clean-up column with an additional acetonitrile elution step (8 mL), iv) centrifugal filtration and v) TurboFlow™ on-line clean-up.

Matrices	Analyte	Concentration (µg/kg)	Recovery (%) [α]				
			i)	ii)	iii)	iv)	v)
Barley	DON	200	96.9 ± 8.4	78.1 ± 7.7	94.2 ± 3.4	96.6 ± 9.8	83.0 ± 7.6
		750	98.6 ± 3.0	73.7 ± 9.4	91.1 ± 10.9	81.9 ± 12.5	72.9 ± 10.1
	DON3Glc	50	97.1 ± 4.3	< 10	28.1 ± 5.3	78.2 ± 7.9	83.0 ± 7.6
		160	93.9 ± 2.5	< 10	20.0 ± 15.6	93.5 ± 12.3	95.8 ± 8.0
Wheat	DON	200	99.3 ± 5.1	98.0 ± 3.5	103.2 ± 7.8	98.4 ± 5.5	101.7 ± 12.4
		750	89.8 ± 9.8	87.1 ± 6.5	104.7 ± 3.9	93.1 ± 12.5	93.1 ± 12.5
	DON3Glc	50	86.6 ± 3.3	< 10	14.6 ± 9.7	85.4 ± 8.9	80.7 ± 9.1
		160	98.3 ± 1.2	< 10	28.8 ± 12.2	90.6 ± 7.6	71.8 ± 5.0

[α] Mean ± RSD of six replicates.

LOD (LOQ = 2.5 x LOD) values for DON ranged between 0.5–1.3 (1.3–3.3) µg/kg and 0.4–1.1 (1.0–2.8) µg/kg in barley and wheat, respectively. For DON3Glc, LODs (LOQs) were 0.3–1.1 (0.8–2.8) µg/kg in barley and 0.1–0.8 (0.3–2.0) µg/kg in wheat. In all cases, the lowest LOD and LOQ values were obtained with TurboFlow chromatography, because analytes were effectively isolated from the bulk of interfering matrix components. Purification of the cereal extracts with MycoSep 227 clean-up columns yielded somewhat comparable LODs/LOQs to those of TurboFlow. However, the additional acetonitrile elution step probably released or pushed through more matrix components from the packing material, resulting in slightly increased LOD and LOQ values for both sample types.

5.2 Multi-mycotoxin determination by LC–MS/MS (II)

5.2.1 Method development and validation

An LC–ESI–TQ–MS/MS method was developed and validated for simultaneous quantification in barley, oats and wheat of five *Fusarium* mycotoxins, HT2, T2, DON, NIV and ZEN, as well as a number of their modified forms: 3Ac-DON, DON3Glc, HT2-3-Glc, NIV3Glc, ZEN14Glc, ZEN14Sulf, ZEN16Glc, α-ZEL, α-ZEL14Glc, β-ZEL and β-ZEL14Glc (Study II). Following method development, in-house validation was performed according to the performance characteristics presented in Commission Regulation (EC) No 401/2006 and Commission Decision 657/2002/EC. Validation was carried out for all three matrices (barley, oats and wheat), which were spiked with low, medium or high analyte mixtures (analyte concentrations in Table 1 of Study II).

Due to the presence of isomeric structures among the target analytes (e.g. α-ZEL/β-ZEL and ZEN14Glc/ZEN16Glc), proper chromatographic separation was essential for accurate quantification. After some necessary adjustments in the chromatographic parameters and the gradient elution programme, satisfactory retention and separation of all analytes were achieved using a Waters Atlantis column. Elution of analytes started at 6.62 min with NIV3Glc and concluded at 14.58 min with ZEN (Figure 8).

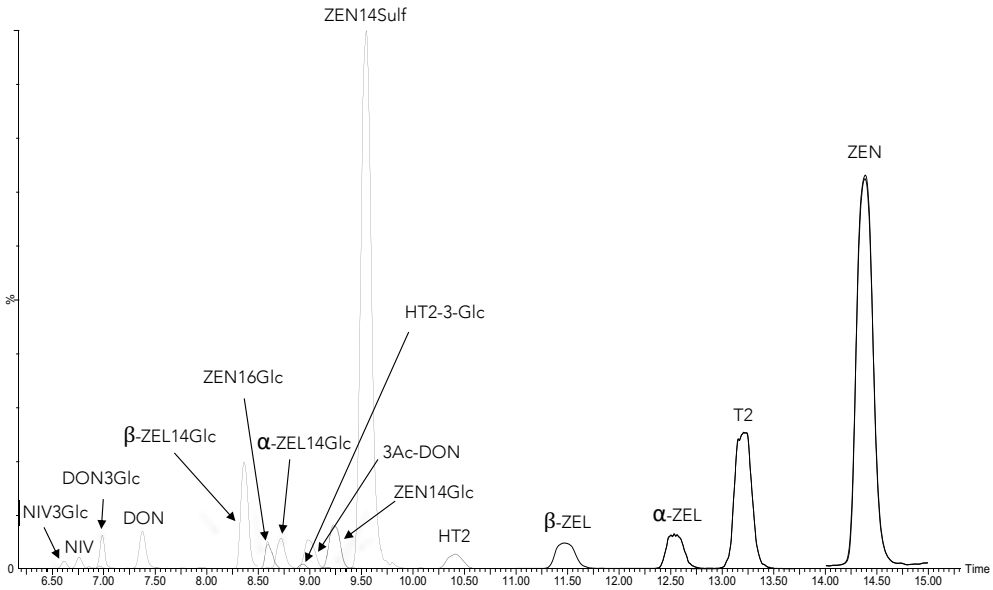


Figure 8. Overlaid quantifier extracted ion chromatograms of a wheat sample spiked with target analytes and analysed with LC–ESI–TQ–MS/MS.

Optimisation was also performed in terms of extraction and more specifically the composition of the extraction solution. Four extraction mixtures were tested, including i) acetonitrile:water:acetic acid (79:20:1, v/v/v), ii) acetonitrile:water:formic acid (79:20.9:0.1, v/v/v), iii) acetonitrile:water (84:16, v/v) and iv) methanol:water (84:16, v/v). Barley (n = 3) and wheat (n = 3) were spiked with 500 µg/kg of each of the native mycotoxins and 3Ac-DON, DON3Glc, α-ZEL and β-ZEL. Mixture i) was demonstrated to be the most efficient in extracting these analytes from cereal matrices, with recoveries of ≥ 90% for native mycotoxins and ≥ 80% for modified mycotoxins.

The five-point calibration curves were linear (R^2 = 0.982–1.000) for all analytes in their respective working ranges. In those samples for which analyte concentrations exceeded the upper spiking level of the calibration curve, appropriate dilutions with blank extracts were prepared and samples were re-analysed. LODs ranged from 0.1 to 4.1 µg/kg in barley, 0.1 to 5.3 µg/kg in oats and 0.1 to 4.3 µg/kg in wheat. In general, DON, T2 and the derivatives of ZEN had the lowest LOD values in all matrices. On the other

hand, 3Ac-DON, HT2-3-Glc and NIV3Glc gave the highest LODs (more detailed information in Study II and its electronic supplementary material).

The R_A varied between 90–116%, 84–115% and 93–112% in barley, oats and wheat, respectively. For the analytes that had legislative performance criteria available, the R_A values were within the typically acceptable 70–120% range. For the remaining analytes with no such criteria, acceptable R_A values were also achieved. RSD_r and RSD_R were also calculated for the three concentration levels. The RSD_r values were relatively high for NIV3Glc and HT2-3-Glc, but never exceeded the 40% limit set in Commission Regulation (EC) No 401/2006 for the native HT2 at the low concentration level (100–200 µg/kg). The RSD_R values for all analytes were also below the 40–60% limit required by the same regulation for the analysis of native DON, HT2, T2 and ZEN. Finally, matrix effects (expressed as SSE) ranged between 59% and 121% for barley, 53% and 122% for oats and 67% and 123% for wheat. The highest signal suppression was observed for ZEN14Glc (SSE = 53% in oats) and the highest signal enhancement for 3Ac-DON (SSE = 123% in wheat).

5.2.2 Survey data of *Fusarium* (modified) mycotoxins

The results of this survey indicated that the most prevalent native mycotoxin was DON (incidence 93% of the samples analysed), followed by NIV (63%) and HT2 (57%). Detailed survey results, including the percentage of samples with mycotoxin or modified mycotoxin concentrations > LOD, as well as average, 95th percentile and maximum concentrations for all target analytes determined in barley, oats and wheat can be found in Table 4 of Study II. Figure 9 presents an overview of the number of samples found contaminated with target analytes concentrations > LOD during this survey.

All of the oat samples and 97% of the wheat samples contained DON above the LOD values, with the mean DON concentrations generally being high in oats (2690 µg/kg) and in wheat (866 µg/kg), whilst in barley the mean values were lower (234 µg/kg). Increased DON concentrations were mostly recorded in oats, but also in a few barley and wheat samples that were cultivated in areas 4 and 7–11 (Figure 6). The legislative MLs for DON for unprocessed oats (1750 µg/kg) and wheat (1250 µg/kg) placed on the market for first-stage processing (Commission Regulation (EC) No 1881/2006) were exceeded in 32% and 23% of the samples, respectively; no barley sample exceeded the corresponding ML for DON. For oats, according to the information provided by the farmers, only three of the samples exceeding the ML were intended for food, and in those, DON levels were between 2440–7280 µg/kg. Two samples that were intended for use as feed were above the guidance value of 8000 µg/kg for products intended for animal feed (Commission Recommendation 2006/576/EC), with considerably elevated DON levels of 16,400 and 23,800 µg/kg.

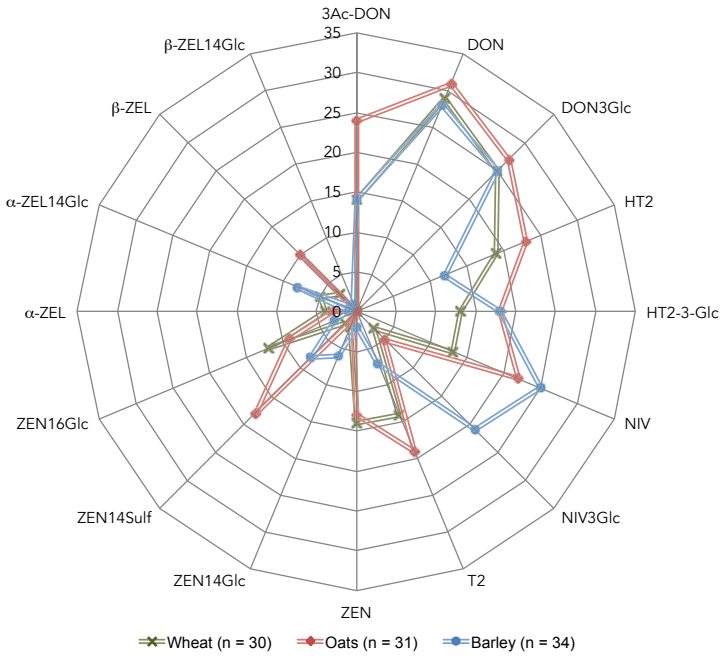


Figure 9. Overview of the number of cereal grains (n = 95), harvested in Finland during 2013, contaminated (> LOD) with *Fusarium* mycotoxins and modified mycotoxins.

In oats, the incidence of 3Ac-DON (up to 2720 µg/kg) was 77.4% and there was a clear correlation between its presence and high DON concentrations. The mean concentration of NIV in oats was 635 µg/kg, with four samples being highly contaminated (> 1400 µg/kg), whereas the average concentrations in barley and wheat were 96.6 and 48.9 µg/kg, respectively. HT2 and T2 were present in higher concentrations in oats (maximum concentrations 1830 µg/kg for HT2 and 548 µg/kg for T2), especially in samples from the southern parts of the country, whereas only relatively low amounts of these mycotoxins were detected in the other two cereal grains. Only one oat sample exceeded the indicative level for the sum of HT2 and T2 (2000 µg/kg; Commission Recommendation 2006/576/EC) for cereal products intended for feed and compound feed, as specified in Recommendation 2013/165/EU, and no cereal samples were above their respective indicative values for unprocessed cereals (100–1000 µg/kg). Only one wheat sample exceeded the ML set for ZEN (100 µg/kg) for cereal grains intended for food, which was also quite heavily contaminated with DON (1660 µg/kg). One oat sample (intended for feed) was found to contain the highest ZEN concentration (675 µg/kg) but was below the guidance value of 2000 µg/kg for animal feed.

All modified mycotoxins analysed with this method were present in at least one sample. The most frequently detected modified mycotoxin by a considerable margin was DON3Glc, which was found in 81% of the barley, oat and wheat samples. It was quantified in concentrations up to 6600 µg/kg in a highly DON-contaminated oat sample. HT2-3-

Glc (incidence 52% of the samples analysed), NIV3Glc (31%), ZEN14Sulf (29%) and ZEN16Glc (25%) were the other modified mycotoxins present in a high proportion of samples. Of these, the highest maximum concentrations were found for ZEN14Sulf, with 220 µg/kg in oats, followed by NIV3Glc, with 65.3 µg/kg in barley. It is worth mentioning that the recently reported ZEN16Glc was detected in more samples than ZEN14Glc, which until a few years ago was the only glucosylated form of ZEN that had been characterised. The other ZEN derivatives (ZEN14Glc, α-ZEL, α-ZEL14Glc, β-ZEL and β-ZEL14Glc) were found in less than 15% of the samples and at low levels. The relative proportions of modified/native mycotoxins, expressed as a molar ratio, were generally between 15–40% for DON3Glc/DON and somewhat higher for HT2-3-Glc/HT2 (30–55%). However, barley samples overall contained higher relative proportions of the modified mycotoxins DON3Glc and HT2-3-Glc, in comparison to the other types of cereal grains. Notably, the DON3Glc/DON ratio reached levels of up to 90%.

5.3 Metabolism of HT2 and T2 in barley and wheat (III & IV)

5.3.1 Metabolite annotation and identification

The qualitative screening of *in planta* HT2 and T2 metabolites was performed by treating of flowering barley and wheat ears with 1:1 mixtures of non-labelled and uniformly ^{13}C-labelled toxins, followed by LC–HRMS/MS analysis and processing with MetExtract. Processing of LC–Orbitrap and LC–QTOF–MS full scan data by MetExtract revealed several individual features that were automatically grouped into distinct chromatographic peaks according to retention time and peak shape similarity. Each feature group represented a distinct metabolite, either including the intact parent toxin or a fragment of it. Figure 10 presents the TIC and the EICs of a barley sample treated with a 1:1 mixture of non-labelled and U-[$^{13}C_{22}$] HT2. The sample was analysed, as part of Study III, with LC–Orbitrap in fast polarity switching mode and processed by MetExtract.

Annotation of *in planta* formed metabolites was primarily based on accurate *m/z* values and LC–HRMS/MS spectral interpretation, but additional information such as assumed ion species and the ^{13}C count were also taken into account. Wherever analytical standards were available (e.g. HT2-3-Glc and 3Ac-T2), their retention time, accurate mass and LC–HRMS/MS spectra were compared with the detected biotransformation products for identification. The majority of biotransformation products could be detected in both polarities, with $[M+Na]^+$, $[M+NH_4]^+$ and $[M+HCOO]^-$ being the most frequently observed ion species in full scan mass spectra. Furthermore, for each metabolite recorded by MetExtract, the number of C atoms was determined, originating from the chemical

backbone of the inoculated precursors HT2 or T2. Structure identification of the putative metabolites designated by MetExtract was performed with targeted LC–HRMS/MS measurements. It is noted that for a small number of metabolites that had been previously reported in the literature and were not picked up by MetExtract, mainly due to low abundance in some samples, they were manually extracted by searching for their EICs of the corresponding ^{12}C and ^{13}C mass signals.

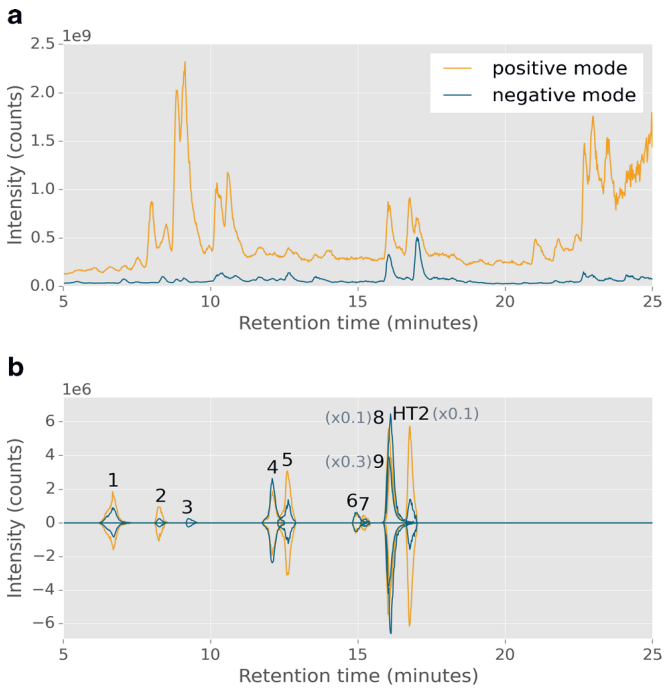


Figure 10. Illustration of (a) fast polarity switching measurement using an Exactive™ Plus Orbitrap instrument and (b) EICs based on MetExtract data processing output. One representative barley sample treated with a 1:1 mixture of non-labelled and uniformly ^{13}C-labelled HT2 (5 time points per flowering ear) was used to depict the positive (orange colour) and negative (blue colour) TIC of LC–Orbitrap measurement and EICs of non-labelled (up) and labelled (down) HT2 and its feature groups (metabolites) obtained by MetExtract software. Numbers above EICs refer to HT2 metabolites listed in Table 6; some EICs were scaled down for better visibility of the low abundant metabolites.

Spectral interpretation was conducted by identifying the characteristic fragments of precursor mycotoxins, calculating molecular formulas and investigating the presence of typical plant conjugates. Mass deviations of precursor ions did not exceed ± 5 ppm in any of the measurements performed by LC–Orbitrap or LC–QTOF–MS. Table 5 provides the main characteristic fragments of the precursor ion of T2 that were used for structure annotation. MS fragments belonging to moieties such as glucose, malonic acid and ferulic acid did not contain a ^{13}C skeleton, thus making it easier to calculate the masses of native plant conjugates; typical fragments of these moieties were also used for metabolite annotation.

Table 5. Characteristic LC–HRMS/MS fragment ions of T2 that were used for structure annotation.

m/z	Ion	Molecular formula
365.1595	[T2 – isovaleric acid + H]$^+$	$C_{19}H_{24}O_7$
305.1383	[T2 – isovaleric acid – acetic acid + H]$^+$	$C_{17}H_{20}O_5$
245.1172	[T2 – isovaleric acid – 2 acetic acid +H]$^+$	$C_{15}H_{16}O_3$
215.1067	[T2 – isovaleric acid – 2 acetic acid – CH_2O +H]$^+$	$C_{14}H_{14}O_2$
185.0961	[$C_{13}H_{12}O$ + H]$^+$	$C_{13}H_{12}O$

One of the novel metabolites found only in T2-treated ears of both cereals was putatively annotated as feruloyl-T2. Two distinct EIC peaks with the same accurate mass but with a slightly different retention time were detected, indicating the presence of *cis*- and *trans*-ferulic acid conjugates. An alternative explanation would be conjugation of T2 to *trans*-ferulic acid and iso-ferulic acid. Figure 11 depicts the fragmentation pattern of one of the isomers of feruloyl-T2. In addition to typical T2 fragments, mass signals of *m/z* 177.0549, *m/z* 145.0279 and *m/z* 117.0330 corresponding to [ferulic acid – H_2O + H]$^+$, [ferulic acid – H_2O – CH_3OH + H]$^+$ and [ferulic acid – H_2O – acetic acid + H]$^+$ were detected. From the SIL data obtained, the T2 structure was reported as fully preserved, and it thus became clear that the ferulic acid was conjugated to the C-3 position of T2. LC–HRMS/MS spectra for the other *in planta* formed metabolites of HT2 and T2 are provided in papers III and IV, or in their electronic supplementary materials, and are not presented here.

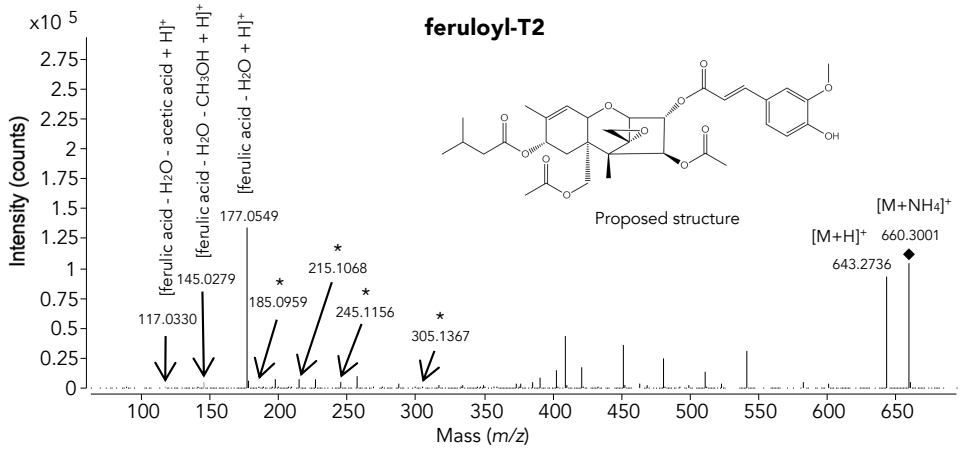


Figure 11. LC–HRMS/MS spectrum (with proposed structure) of feruloyl-T2, an *in planta* metabolite of T2. The ammonium adduct was chosen as the precursor (marked with a diamond). Characteristic T2 fragments (Table 5) are marked with an asterisk (*).

Identified and annotated metabolites in barley are summarised in Tables 6 (for HT2; n = 9) and 7 (for T2; n = 13). All nine modified mycotoxins detected in HT2-treated barley ears were also identified in the T2-treated samples, due to the fast conversion of HT2 to T2, in addition to HT2, T2-α-Glc, 3Ac-T2 and two isomers of feruloyl-T2. The

accurate mass and ion species of the chosen precursor were used for targeted LC–HRMS/MS analysis, except for metabolites in very low abundances (marked with ***), for which it was not possible to obtain a clear spectrum.

Identically to the barley metabolomics experiments, raw LC–QTOF–MS data derived from the analysis of wheat samples inoculated with either HT2 or T2 were processed by MetExtract and inspected for *in planta* formed metabolites. For paper IV, only data from LC–QTOF–MS instrumentation and without fast polarity switching were obtained. In total, 31 feature groups were identified in wheat, out of which 12 in the HT2-treated samples and 13 in the T2-treated samples were of sufficient intensity to enable further structural characterisation by LC–HRMS/MS measurements; no ion pairs were detected in the mock samples. The LC–HRMS/MS spectra of the detected metabolites of HT2 and T2 in wheat were compared with those observed in barley and were found to be identical.

Table 6. Overview of putative HT2 metabolites detected in barley ears of the variety Calcule. Samples were collected after treatment with a mixture of 200 µg/ear $^{12}C/^{13}C$ HT2 (1:1) during days 1, 2, 3, 5, and 7 on the same ear. Identification and annotation were performed with accurate mass measurements and LC–HRMS/MS spectra.

Peak No.	RT (min)	Molecular formula [α]	Ion species [β]	*m/z* [β]	Mass accuracy (ppm)	^{13}C count	Putative metabolite
1	6.7	$C_{23}H_{34}O_{12}$	$[M+NH_4]^+$	520.2380	−1.6	17	15-acetyl-T2-tetraol-glucoside**
2	8.3	$C_{26}H_{36}O_{15}$	$[M+NH_4]^+$	606.2381	−1.9	17	15-acetyl-T2-tetraol-malonyl-glucoside**
3	9.2	$C_{23}H_{32}O_{12}$	$[M+HCOO]^-$	545.1867	−1.6	17	15-acetyl-T2-tetraol-glucoside[−2H]***
4	12.1	$C_{28}H_{42}O_{14}$	$[M+NH_4]^+$	620.2900	−2.1	22	hydroxy-HT2-glucoside**
5	12.6	$C_{31}H_{44}O_{17}$	$[M+NH_4]^+$	706.2906	−1.5	22	hydroxy-HT2-malonyl-glucoside**
6	15.0	$C_{26}H_{40}O_{12}$	$[M+Na]^+$	567.2402	−1.8	20	T2-triol-glucoside**
7a	15.2	$C_{34}H_{52}O_{18}$	$[M+HCOO]^-$	793.3124	−1.5	22	HT2-di-glucoside**
7b	15.8	$C_{34}H_{52}O_{18}$	$[M+HCOO]^-$	793.3124	−1.5	22	HT2-di-glucoside**
8	16.1	$C_{28}H_{42}O_{13}$	$[M+NH_4]^+$	604.2950	−2.3	22	HT2-3-Glc*
9	16.1	$C_{31}H_{44}O_{16}$	$[M+NH_4]^+$	690.2950	−2.6	22	HT2-malonyl-glucoside**
HT2	16.8	$C_{22}H_{32}O_8$	$[M+NH_4]^+$	442.2428	−1.7	22	HT2*

[α] Molecular formula of neutral compound.

[β] Ion species and accurate mass of the most abundant ion.

* Confirmation with standard by comparison of retention time, accurate mass and LC–HRMS/MS spectra.

** Annotation with accurate mass and LC–HRMS/MS spectra.

*** Annotation with accurate mass.

Table 7. Overview of putative T2 metabolites detected in barley ears of the variety Calcule. Samples were collected after treatment with a mixture of 200 µg/ear $^{12}C/^{13}C$ T2 (1:1) during days 1, 2, 3, 5, and 7 on the same ear. Identification and annotation were performed with accurate mass measurements and LC–HRMS/MS spectra. Peaks i–iv and T2 are not shown in Figure 10.

Peak No.	RT (min)	Molecular formula [α]	Ion species [β]	*m/z* [β]	Mass accuracy (ppm)	^{13}C count	Putative metabolite
1	6.7	$C_{23}H_{34}O_{12}$	$[M+NH_4]^+$	520.2384	−0.9	17	15-acetyl-T2-tetraol-glucoside**
2	8.3	$C_{26}H_{36}O_{15}$	$[M+NH_4]^+$	606.2382	−1.7	17	15-acetyl-T2-tetraol-malonyl-glucoside**
3	9.3	$C_{23}H_{32}O_{12}$	$[M+HCOO]^-$	545.1872	−0.7	17	15-acetyl-T2-tetraol-glucoside[−2H]***
4	12.1	$C_{28}H_{42}O_{14}$	$[M+NH_4]^+$	620.2902	−1.7	22	hydroxy-HT2-glucoside**
5	12.6	$C_{31}H_{44}O_{17}$	$[M+NH_4]^+$	706.2912	−0.7	22	hydroxy-HT2-malonyl-glucoside**
6	14.9	$C_{26}H_{40}O_{12}$	$[M+HCOO]^-$	589.2500	−0.3	20	T2-triol-glucoside**
7a	15.2	$C_{34}H_{52}O_{18}$	$[M+HCOO]^-$	793.3139	+0.4	22	HT2-di-glucoside**
7b	15.8	$C_{34}H_{52}O_{18}$	$[M+HCOO]^-$	793.3139	+0.4	22	HT2-di-glucoside**
8	16.1	$C_{28}H_{42}O_{13}$	$[M+NH_4]^+$	604.2953	−1.8	22	HT2-3-Glc*
9	16.1	$C_{31}H_{44}O_{16}$	$[M+NH_4]^+$	690.2952	−2.3	22	HT2-malonyl-glucoside**
HT2	16.8	$C_{22}H_{32}O_8$	$[M+NH_4]^+$	442.2428	−1.7	22	HT2*
i	17.0	$C_{30}H_{44}O_{14}$	$[M+Na]^+$	651.2617	−1.0	24	T2-Glc***
T2	17.9	$C_{24}H_{34}O_9$	$[M+NH_4]^+$	484.2532	−1.9	24	T2*
ii	19.1	$C_{26}H_{36}O_{10}$	$[M+NH_4]^+$	526.2640	−1.3	24	3Ac-T2*
iii	20.0	$C_{34}H_{42}O_{12}$	$[M+Na]^+$	665.2557	−1.7	24	feruloyl-T2**
iv	20.2	$C_{34}H_{42}O_{12}$	$[M+Na]^+$	665.2551	−2.6	24	feruloyl-T2**

[α] Molecular formula of neutral compound.

[β] Ion species and accurate mass of the most abundant ion.

* Confirmation with standard by comparison of retention time, accurate mass and LC–HRMS/MS spectra.

** Annotation with accurate mass and LC–HRMS/MS spectra.

*** Annotation with accurate mass.

All putative biotransformation products detected in wheat after treatment of wheat ears with either HT2 or T2 are illustrated as EICs in Figure 12. In general, the *in planta* metabolism of HT2 and T2 was closely related between barley and wheat. There were several common metabolites, with the exception of 15-acetyl-T2-tetraol-malonyl-glucoside and 15-acetyl-T2-tetraol-glucoside[–2H], which were only annotated in barley, and dehydro-HT2-glucoside, which was only found in wheat. However, probably the most striking difference was the formation of two metabolites following exposure to HT2, which were only found in wheat and were putatively annotated as 3-acetyl-HT2 and T2. It should be noted, however, that the peak corresponding to the latter structure could have originated from the second isotopologue of ^{13}C-T2, which could have been present as an impurity of the ^{13}C-HT2 used for the treatment of the ears. Thus, the formation of T2 by wheat cannot be confirmed with certainty based on the available data. A summary of the identified and annotated metabolites of HT2 and T2 in the wheat variety Remus is presented in Table 8.

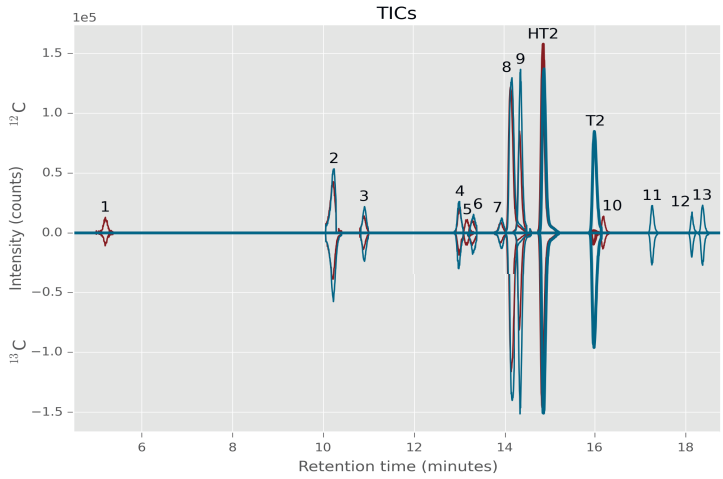


Figure 12. Overlaid EICs based on MetExtract data processing output showing the biotransformation products of one wheat sample treated with a mixture of $^{12}C/^{13}C$ HT2 (red colour) and one treated with a mixture of $^{12}C/^{13}C$ T2 (blue colour). EICs of non-labelled metabolites are displayed with positive intensity values, and those of the corresponding labelled metabolites as negative intensities. Numbers above EICs refer to HT2 and T2 metabolites listed in Table 8.

Table 8. Overview of putative HT2 and T2 metabolites detected in wheat ears of the variety Remus (susceptible to FHB). Samples were collected after treatment with a mixture of either 200 µg/ear $^{12}C/^{13}C$ HT2 (1:1) or 200 µg/ear $^{12}C/^{13}C$ T2 (1:1) during days 1, 2, 3, 5 and 7 on the same ear. Identification and annotation were performed with accurate mass measurements and LC–HRMS/MS spectra. Metabolites 11–13 were only detected in samples treated with T2.

Peak No.	RT (min)	Molecular formula [α]	Ion species [β]	*m/z* [β]	Mass accuracy (ppm)	^{13}C count	Putative metabolite
1	5.2	$C_{23}H_{34}O_{12}$	$[M+Na]^+$	525.1940	−0.41	17	15-acetyl-T2-tetraol-glucoside***
2	10.2	$C_{28}H_{42}O_{14}$	$[M+Na]^+$	625.2461	−0.92	22	hydroxy-HT2-glucoside**
3	10.9	$C_{31}H_{44}O_{17}$	$[M+Na]^+$	711.2447	−3.33	22	hydroxy-HT2-malonyl-glucoside**
4	13.0	$C_{26}H_{40}O_{12}$	$[M+HCOO]^-$	589.2498	−0.64	20	T2-triol-glucoside**
5	13.2	$C_{28}H_{40}O_{13}$	$[M+HCOO]^-$	629.2446	−0.78	22	dehydro-HT2-glucoside**
6	13.3	$C_{34}H_{52}O_{18}$	$[M+HCOO]^-$	793.3135	−0.08	22	HT2-di-glucoside**
7	14.0	$C_{34}H_{52}O_{18}$	$[M+HCOO]^-$	793.3135	−0.08	22	HT2-di-glucoside**
8	14.1	$C_{28}H_{42}O_{13}$	$[M+Na]^+$	609.2518	+0.00	22	HT2-3-Glc*
9	14.3	$C_{31}H_{44}O_{16}$	$[M+Na]^+$	695.2505	−2.39	22	HT2-malonyl-glucoside**
HT2	14.9	$C_{22}H_{32}O_8$	$[M+Na]^+$	447.1992	+0.59	22	HT2*
T2	16.0	$C_{24}H_{34}O_9$	$[M+Na]^+$	489.2098	+0.68	22	T2*
10	16.2	$C_{24}H_{34}O_9$	$[M+Na]^+$	489.2083	−2.46	22	3-acetyl-HT2**
11	17.3	$C_{26}H_{36}O_{10}$	$[M+Na]^+$	531.2187	−2.57	24	3Ac-T2*
12	18.1	$C_{34}H_{42}O_{12}$	$[M+Na]^+$	665.2561	−1.12	24	feruloyl-T2**
13	18.4	$C_{34}H_{42}O_{12}$	$[M+Na]^+$	665.2561	−1.12	24	feruloyl-T2**

[α] Molecular formula of neutral compound.

[β] Ion species and accurate mass of the most abundant ion.

* Confirmation with standard by comparison of retention time, accurate mass and LC–HRMS/MS spectra.

** Annotation with accurate mass and LC–HRMS/MS spectra.

*** Annotation with accurate mass.

5.3.2 Time course kinetics

The kinetic profiles of T2 and HT2 and their formed metabolites were studied in the barley variety Calcule and in the wheat lines Remus (susceptible to FHB) and CM-82036 (resistant to FHB). Treated ears of the plants did not show any premature bleaching after a single exposure of the mycotoxins. The absolute concentration values of HT2, HT2-3-Glc and T2 were quantified with analytical standards, corrected for the observed SSE at the respective dilution, corrected for the ear weight and are presented here in µmol/treated ear. For quantification, the theoretically added mycotoxin amounts at Day 0 of 200 µg HT2 (equal to 0.471 µmol) and 200 µg T2 (0.429 µmol) were used instead of the measured concentration. This adjustment was necessary due to the poor diffusion into the plant cells of the toxins immediately after treatment.

Figure 13 illustrates the quantified time courses of HT2, T2 and their major modified form, HT2-3-Glc, in barley and the two wheat varieties. For all other metabolites, data obtained from EIC integrated peak areas were corrected for the individual ear weight and correlated with the highest observed area; their kinetic graphs are presented in papers III and IV as relative abundances.

In barley, only about 25% of the inoculated HT2 remained unmodified within the first 24 h following treatment, with more than half of it being transformed into HT2-3-Glc. A steady decrease of HT2 and HT2-3-Glc was observed until the end of the experiment (Ripen), resulting in a final content of 7% and 34%, respectively, relative to the originally inoculated toxin. A similarly rapid decrease of HT2 occurred during the first day in both Remus and CM-82036 wheat varieties. However, HT2-3-Glc reached its peak concentration at a later time point, between days 3 and 7 after treatment. HT2-3-Glc concentrations were in all cases lower at ripening compared to the maximal amounts, a fact that implied further metabolism of this metabolite into additional biotransformation products such as HT2-di-glucoside, HT2-malonyl-glucoside, hydroxy-HT2-glucoside, hydroxy-HT2-malonyl-glucoside and 15-acetyl-T2-tetraol-glucoside, which reached maximal abundance at the ripening stage. In the wheat kinetics experiments, no significant differences in the amounts of HT2 and T2 metabolites in the two wheat varieties were determined.

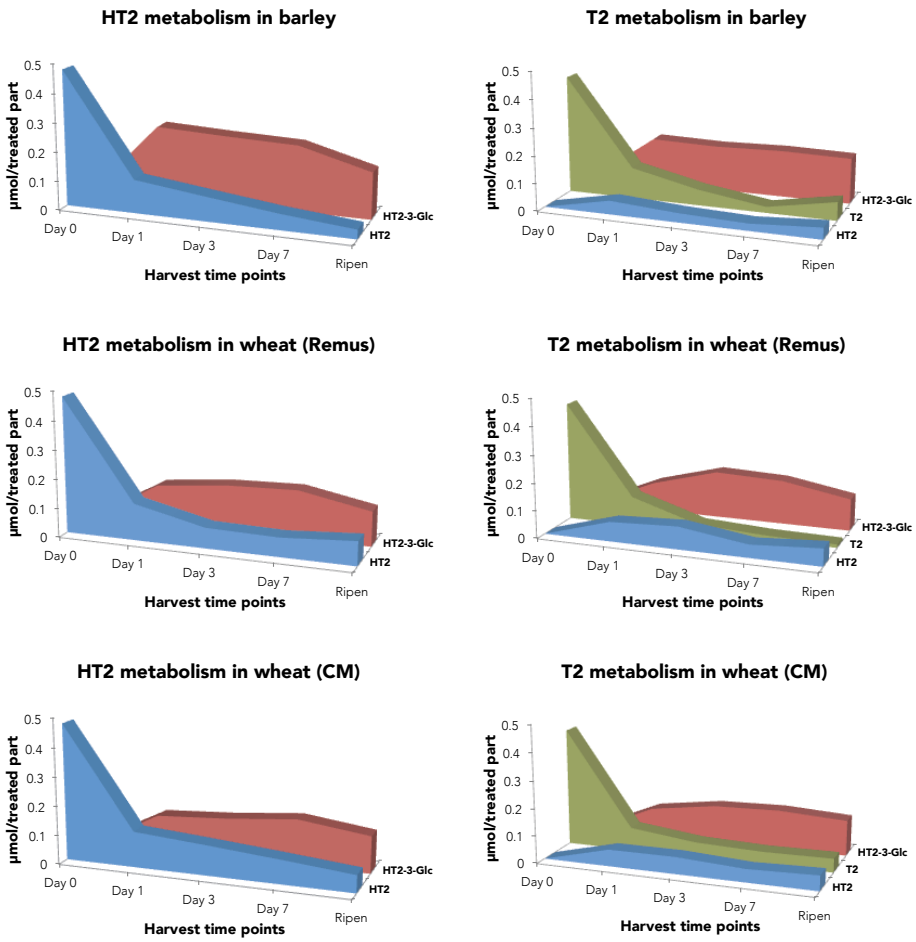


Figure 13. Quantified time course kinetics of HT2 (blue colour), T2 (green colour) and HT2-3-Glc (red colour) in the barley variety Calcule and in the wheat lines Remus (susceptible to FHB) and CM-82036 (resistant to FHB). Ears were treated with either 200 µg HT2 or 200 µg T2 and harvested immediately, 1, 3 and 7 days after treatment and at the full-ripening stage (Ripen).

In T2-treated ears, de-acetylation of T2 to HT2 progressed rapidly, and after one day, only 26%, 25% and 20% of the initially added toxins could be determined in barley, and the Remus and CM-82036 wheat varieties, respectively. At the same time HT2 was rapidly glucosylated to HT2-3-Glc and other metabolic products, as was shown in the HT2-treated samples. For instance, barley had already biotransformed 52% of the initially inoculated T2 into HT2 and HT2-3-Glc within one day. Besides glucosylation, C-3 acetylation and (iso)ferulic acid conjugation were the immediate responses of plant detoxification mechanisms against T2. However, these compounds were further metabolised or became covalently bound to plant matrix, as their relative concentrations decreased over time. Conjugations with an additional glucose moiety or malonic acid to

HT2 mono-glucosides occurred later. Finally, hydrolysis of the acetyl group of HT2-3-Glc in C-15, leading to the formation of T2-triol-glucoside, presumably constitutes a consecutive reaction, because the compound was only observed in the ripened samples. Figure 14 depicts the integrated proposed metabolic fate of HT2 and T2 in barley and wheat based on the findings presented in Studies III and IV.

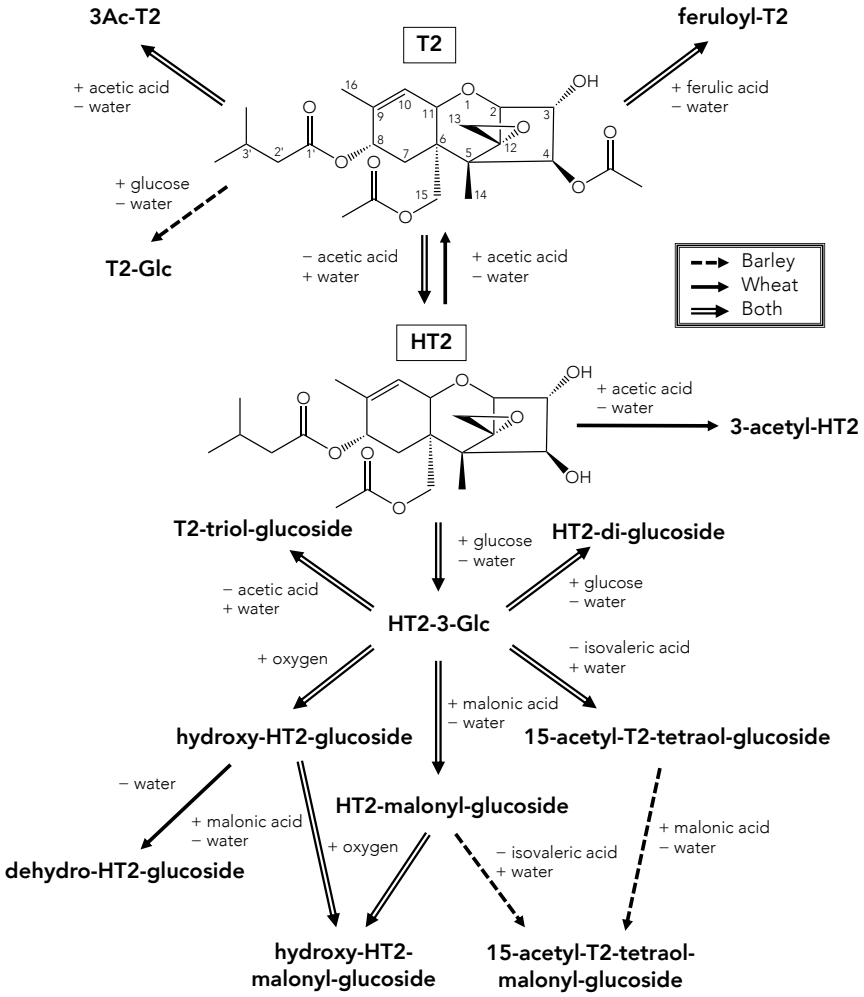


Figure 14. Proposed integrated metabolic pathways of HT2 and T2 in barley and wheat.

In summary, the *in planta* metabolism of HT2 and T2 in barley and wheat was unravelled by SIL-assisted metabolomics experiments and time course kinetic studies. Exposure to HT2 and T2 primarily activated metabolic reactions involving hydroxylation, (de)acetylation and various conjugations with glucose, malonic acid and ferulic acid. Kinetic data revealed that HT2 was the main metabolite of T2, whilst HT2-3-Glc was rapidly formed in high amounts, regardless of whether plants are exposed to HT2 or T2.

5.4 Yeast–mycotoxin interactions during beer fermentation (V)

In Study V, two 96-h brewing fermentation experiments were performed using A15 yeast exposed to DON or DON3Glc, as well as HT2 and/or T2. In the first experiment, A15 yeast was treated with either 100 µg/L (low dose groups) or 10,000 µg/L (high dose groups) DON, HT2 or T2. For Experiment 2, 400 µg/L DON3Glc or a mixture of 5000 µg/L HT2 and 5000 µg/L T2 was used. The influence of these toxins on viability, fresh yeast mass, Plato, pH and alcohol production in comparison to the control groups is presented in Table 9.

Table 9. Influence of DON, HT2 and T2 at low (100 µg/L, n = 3) and high (10,000 µg/L, n = 3) concentrations (Experiment 1), as well as DON3Glc (400 µg/L, n = 3) and a mixture of HT2 and T2 (5000 µg/L each, n = 3) (Experiment 2) on the growth, viability and fermentation of sugars after four-day brewing fermentations by the lager yeast strain A15 in 11.5° Plato wort.

	Toxin	Concentration (µg/L)	Viability (%)	Fresh yeast mass (g/L)	Plato	pH	Alcohol (v/v)
Experiment 1	Control	0	99.5 ± 0.1	41.4 ± 0.2	3.39 ± 0.01	3.87 ± 0.01	3.21 ± 0.04
	DON	100	99.6 ± 0.1	38.9 ± 1.6	3.38 ± 0.08	3.86 ± 0.01	3.23 ± 0.11
		10,000	99.6 ± 0.1	36.7 ± 1.7	3.40 ± 0.03	3.88 ± 0.02	3.16 ± 0.04
	HT2	100	99.5 ± 0.1	38.1 ± 1.1	3.39 ± 0.06	3.86 ± 0.01	3.19 ± 0.08
		10,000	99.5 ± 0.1	41.8 ± 0.8	3.39 ± 0.06	3.85 ± 0.01	3.14 ± 0.09
	T2	100	98.3 ± 1.4	41.4 ± 0.3	3.37 ± 0.05	3.85 ± 0.02	3.22 ± 0.08
		10,000	95.8* ± 2.2	40.8 ± 1.4	3.25* ± 0.05	3.84 ± 0.01	3.37* ± 0.07
Experiment 2	Control	0	99.4 ± 0.1	35.1 ± 0.2	2.15 ± 0.06	4.15 ± 0.02	3.59 ± 0.08
	DON3Glc	400	99.3 ± 0.1	36.1 ± 0.3	2.28 ± 0.04	4.14 ± 0.01	3.46 ± 0.04
	HT2 + T2	5000 each	98.3* ± 0.1	37.5 ± 1.6	2.32* ± 0.02	4.08* ± 0.01	3.53 ± 0.02

* Statistically significant difference ($p < 0.05$) compared to the control group.

In general, A15 yeast was tolerant even in the dose groups treated with 10,000 µg/L DON, HT2 or T2. The only exposure group showing any statistically significant difference ($p < 0.05$) in comparison to the controls was the one treated with 10,000 µg/L T2. Even in that group, viability (%) was only slightly reduced to 95.8%, compared to approximately 99.5% observed in all other groups. These small deviations in the high T2 group were also reflected in lower values for wort concentrations, 3.25° Plato compared to 3.39° Plato in the control, and in a minor increase in alcohol production, 3.37% *versus* 3.21% in the control. For all other measured parameters, no deviations occurred between treated and control groups. Exposure to 400 µg/L DON3Glc or a mixture of HT2 and T2 (5000 µg/L per toxin) in the second experiment did not result in any substantial adverse effects on the viability of cells or fermentation performance.

The kinetic profiles of the four toxins were also investigated by relating the absolute quantified mean concentrations at time points 4, 24, 28, 48, 72 and 96 h to each

analyte's mean concentration determined at 0 h. In the low exposure groups (100 µg/L), a rapid reduction in the dosed toxins of between 5% and 13% was already observed from the first sampling (time point: 4 h). A statistically significant overall decrease after 96 h fermentation was observed for the type A trichothecenes HT2 (66%) and T2 (69%) compared to DON (85%). Another difference was that for DON, no further reduction occurred after the first 24 h in the low exposure group, whereas for HT2 and T2, the decrease was constant throughout the 96 h of Experiment 1. In the high exposure groups, residual concentrations were consistently higher than in the low exposure groups for all three mycotoxins, resulting in residual relative concentrations between 85% and 91%. Data from Experiment 2 revealed that when HT2 and T2 were inoculated together, in concentrations of 5000 µg/L each, 80% and 78% were retrieved at the end of fermentation, respectively. Wort samples treated with DON3Glc were analysed at the end of the experiment for the presence of its precursor. However, no DON was found, even though the concentration of DON3Glc was reduced by 17% after 96 h fermentation. Therefore, no de-conjugation of the glucose moiety of DON3Glc occurred during yeast fermentation. On the contrary, yeast was found able to glucosylate DON, resulting in the formation of DON3Glc, which was verified by standard measurements (Table 10).

Table 10. Overview of putative metabolites, as provided by MetaboLynx™ XS, that were detected during a 96-h brewing fermentation by the lager yeast strain A15 in an 11.5° Plato wort treated with 10,000 µg/L of DON, HT2 or T2.

Exposure group, detection time	RT (min)	Molecular formula [α]	Ion species [β]	*m/z* [β]	Mass accuracy (mDa)	Putative metabolite
DON, 72 h	6.1	$C_{21}H_{30}O_{11}$	$[M+CH_3COO]^-$	517.1934	+1.4	DON3Glc*
DON, 24 h	7.2	$C_{17}H_{22}O_7$	$[M-H]^-$	337.1245	−4.3	3*/15Ac-DON**
HT2, 72 h	10.4	$C_{22}H_{32}O_{11}S$	$[M+Na]^+$	527.1536	−2.1	HT2-sulfate**
T2, 28 h	10.8	$C_{22}H_{32}O_8$	$[M+NH_4]^+$	442.2426	−0.3	HT2*
HT2, 28 h	12.9	$C_{24}H_{34}O_9$	$[M+NH_4]^+$	484.2535	−0.1	T2*/3-acetyl-HT2**
T2, 4 h	14.6	$C_{26}H_{36}O_{10}$	$[M+NH_4]^+$	526.2639	−0.2	3Ac-T2**

[α] Molecular formula of neutral compound.

[β] Accurate mass and ion species of the most abundant ion.

* Confirmation with standard by comparison of retention time, accurate mass and HRMS/MS-spectra.

** Annotation with accurate mass and HRMS/MS-spectra.

A molar mass balance of the inoculated toxins was calculated after analysis with LC–TQ–MS/MS of wort and yeast biomass, which was collected at the end of the fermentation experiments. To compensate for matrix effects, matrix-assisted calibration with yeast biomass from the control groups was used. In the low-dose groups, target analytes were below LOD values. In the high-dose groups, minor toxin amounts, between 0.3 and 0.8 µg, were determined in yeast biomass. The absolute mycotoxin amounts that were inoculated to wort in the high dose groups were measured to be at the beginning of fermentation: 1120 µg for DON, 1029 µg for HT2 and 893 µg for T2. Following the four-day fermentation, the remaining amounts were 921, 934 and 756 µg for DON, HT2

and T2, respectively. These differences between initial and final concentrations can be attributed to the physical binding and metabolism of the toxins by A15 yeast.

Wort was also analysed for the presence of metabolites formed as a result of DON, DON3Glc, HT2 and T2 biotransformation by yeast during fermentation. Raw full scan LC–QTOF–MS data were processed with MetaboLynx XS software and putative metabolites were annotated or identified by additional LC–QTOF–MS/MS measurements. All putatively identified metabolites are presented in Table 10 and relevant MS/MS spectra are provided in paper V. DON3Glc, 3Ac-DON, HT2 and T2 were detected in samples not inoculated with these toxins and were verified with available analytical standards. For the remaining putative metabolites, which were annotated as 15Ac-DON, HT2-sulfate, 3-acetyl-HT2 and 3Ac-T2 and for which analytical standards were not available, spectral analysis revealed the presence of characteristic fragments belonging to their respective parent toxins. Based on these findings, trichothecenes are primarily metabolised via (de)acetylation, glucosylation and sulfation.

6 DISCUSSION

6.1 Method development

6.1.1 Sample preparation

The choice and optimisation of sample preparation is paramount to the success of any analytical method, from the simplest to the most complex mycotoxin determinations. For the majority of LC–MS/MS analyses, extraction is necessary in order to efficiently isolate compounds of interest from any given matrix. Sample clean-up, however, can be considered as a double-edged sword, because on the one hand it may alleviate the severity of matrix effects, but on the other it may remove analytes of interest (Köppen et al., 2010). In Studies I–V, various extraction procedures were utilised according to the chemical nature of target mycotoxins and derivatives.

Selection of the most appropriate extraction mixture and extraction conditions for a multitude of analytes with largely varying physico-chemical properties is generally challenging. Extraction solutions with a large proportion of methanol or acetonitrile (> 75%), with or without the addition of acid (acetic or formic acid), have been routinely used in most mycotoxin analyses (Capriotti et al., 2012). In the course of this thesis, short extraction trials were conducted whenever necessary to evaluate the efficiency of extraction solutions. Since a number of the analytes that were included in the multi-(modified) mycotoxin method, developed as part of Study II, had never before been quantitatively determined (e.g. NIV3Glc, ZEN16Glc), four different extraction mixtures were assessed for their efficiency in extracting the target analytes. The applicability of the solution acetonitrile:water:acetic acid (79:20:1, v/v/v), which has been employed in other methods based on the dilute and extract approach for analysis of type A/B trichothecenes, ZEN and modified forms thereof in cereal matrices (Vendl et al., 2009; De Boevre et al., 2012; Soleimany et al., 2012), was confirmed. This was primarily due to the improved extraction efficiency that was accomplished. For those methods in which multiple and/or unknown compounds were included, the versatile extraction mixtures of acetonitrile:water:acetic acid (79:20:1, v/v/v) or acetonitrile:water:formic or acetic acid (79:20.9:0.1, v/v/v) were also used. Minor adaptations on a case-by-case basis were implemented to achieve optimal extraction of target analytes and alleviate matrix effects by, for instance, incorporating a de-lipidation step (Study II) or the addition of Celite (Study V).

In the inter-laboratory sample preparation comparison study (I), the extract and shoot approach and TurboFlow chromatography were the only sample preparation techniques that were deemed suitable for the concurrent determination of DON and

DON3Glc and performed in accordance with the performance characteristics set by current legislation. MycoSep 227 and centrifugal filtration required additional steps, frequent system maintenance (e.g. cleaning parts of the ESI interface from matrix components) or were found unsuitable for the simultaneous determination of DON and DON3Glc compounds, which possess different polarities. Whilst MycoSep cartridges have been extensively used since the 1990s for sample purification, especially in trichothecene analysis, they may not always be appropriate for the clean-up of samples containing mycotoxins and derivatives with largely varying polarities. It appears that the more hydrophilic DON3Glc was retained within the column packing material, together with matrix co-extracts, and did not pass through to the supernatant with DON. Even though the 227 cartridge is specifically designed for type A and B trichothecenes, the 226 model or any other purification cartridge from the same manufacturer that is designed to accommodate a broader range of polarities may have been more suitable for DON3Glc.

However, an extensive assessment of eight commercially available clean-up cartridges was performed by Vendl et al., (2009), including SPE, immunoaffinity columns and MycoSep (226 and 230), as well as using primary and secondary amines for clean-up. None of the tested clean-up methods yielded satisfactory results for the analysis of DON, ZEN or eight modified forms, with the authors stating that a group of compounds having such a wide polarity range is unlikely to benefit in its entirety from a single clean-up approach. De Boevre et al. (2012), also tested four clean-up columns (Florisil® Bond Elut, MultiSep® 225, MycoSep 229 and Oasis® HLB), but did not achieve acceptable recoveries for modified mycotoxins, and ended up using the extract/dilute and shoot approach. Nonetheless, in the case of the type A trichothecenes HT2 and T2, Lattanzio et al. (2012) applied the MycoSep 227 column in the clean-up of oat and wheat samples and accomplished the identification of the polar HT2 and T2 glucosides. It should be mentioned, however, that ideally appropriate clean-up would be very beneficial to LC–MS/MS determinations, as alongside the extraction of a wide range of analytes, the co-extraction of bulk matrix constituents is undesirable, because it may reduce the lifetime of the chromatographic column, result in frequent maintenance of the mass spectrometer and interfere with the ionisation process causing analyte SSE effects.

Aside from Study I, which aimed at assessing different sample preparation and clean-up techniques, pre-treatment and purification were in all other studies kept to a minimum. With every clean-up procedure to reduce or eliminate unwanted co-extracted matrix components and minimise matrix effects, certain analyte classes may not be amenable to it and consequently become lost during the process and remain undetectable (Krska et al., 2008). Thus, clean-up was generally omitted in Studies II–V, as certain compounds could have potentially been lost during the process. Moreover, the high selectivity guaranteed by modern mass spectrometers allows the concurrent determination of multiple target analytes with minimal need for clean-up (Sulyok et al.,

2010). As a result, multi-mycotoxin quantitative LC–MS/MS methods and LC–HRMS methods used for metabolomics applications tend to bypass perplexing clean-up procedures and rely on extract/dilute and shoot-type approaches, in which raw extracts are directly injected into the MS.

6.1.2 Method validation and performance characteristics

In principle, validation aims to demonstrate that a method is fit for the intended purpose and can produce selective, sensitive, accurate and reproducible results for a given property. Method validation is a sub-process of the quality assurance scheme of an analytical method (Brera and Miraglia, 1996). All methods developed for the quantification of mycotoxins or modified mycotoxins were in-house validated according to performance criteria and recommendations established in Commission Regulation (EC) No 401/2006 and Commission Decision 2002/657/EC. The degree of validation was dictated by the intended application and the fact that the methods were developed for research purposes and not for official control of mycotoxins and derivatives thereof. Complete validation of all analytes included in the methods was hindered by the unavailability or the very limited amounts of analytical standards for certain modified mycotoxins.

The multi-(modified) mycotoxin method developed in Study II was successfully validated in three matrices (barley, oats and wheat). Calibration curves were linear over the respective working range of each analyte and the R_A values were in agreement with the recommended legislative values (typically 70–120%). The LODs varied considerably, as expected, among different analytes and matrices, but generally allowed for the determination of all analytes at low µg/kg levels. As a general remark, the lowest LODs were achieved for DON, T2 and the derivatives of ZEN in all three matrices. The highest LODs were observed for 3Ac-DON, HT2-3-Glc and NIV3Glc. Since no performance criteria are available for modified forms, RSD_r and RSD_R values were calculated and compared to acceptable limits from their respective precursors DON, HT2, T2 and ZEN, as laid down in Commission Regulation (EC) No 401/2006.

In the same method, minor to moderate matrix effects were documented, with 63% of the target analytes in barley and wheat and half of them in oats being within the 80–120% "acceptable" range. From the same results, oats appear to have caused slightly more pronounced matrix effects in comparison to barley and wheat, and these effects persisted even after de-lipidation. The methods in papers III–V were only validated on the basis of the quantification of target analytes for which analytical standards were available, and not on parameters related to metabolite identification. For these methods, appropriate dilutions of the sample extracts were performed, and absolute concentrations were only corrected for matrix effects if the SSE values were below 85%

or above 115%. Notably, there is no legislatively acceptable range for matrix effects and different authors have applied different ranges, usually between 90–110% (Malachová et al., 2014) or 80–120% (Varga et al., 2013).

Despite the considerable progress of metabolomics and continuous expansion to new applications, major challenges still remain. In addition to fundamental aspects such as the reliable annotation of true sample metabolites, their putative identification and accurate (semi)-quantitation, proper workflow validation should also be addressed (Kluger et al., 2015a). Validation of untargeted methodologies is equally important to that of purely quantitative methods, in order to ensure that reliable and reproducible measurements are obtained. However, the analytical challenges and criteria are very different from quantitative methods. Untargeted metabolomics is intended for metabolite identification within biological samples, and based on this purpose, validation parameters ought to be established (Naz et al., 2014). Regarding the workflow implemented in the untargeted metabolomic Studies III and IV, it would have been very complex and cost-intensive to conduct a comprehensive validation. In 2007, the Metabolomics Standards Initiative (MSI) published the minimum metadata related to chemical analysis aspects of metabolomics as a means to communicate the confidence in identification (Sumner et al., 2007). This concept relies on four levels of identification that have been revised over the years and refined to cover broader applications, although it still lacks the ability to consider all strategies for the identification of metabolites, with new approaches continuously being introduced in this dynamic research field.

6.1.3 Qualitative screening

In Studies III and IV, the formation of T2 and HT2 metabolites in barley and wheat was examined via SIL-assisted untargeted metabolomics, LC–HRMS measurements and data processing with MetExtract software. The formed metabolites consisted of pairs of their native and partly labelled isomers. Most metabolites that were detected, contained, contingent on treatment, the intact chemical backbone of either HT2 or T2. This could be clearly verified by a measurable mass difference of 22.0738 Da, which corresponded to the mass difference between the 22 ^{12}C in the native and the 22 ^{13}C in the fully labelled HT2 tracers. Likewise, a mass difference of 24.0805 Da between the labelled and non-labelled ion pairs of T2 was always recorded in metabolites containing its intact 24-carbon structure. Fragmentation patterns of HT2 and T2 backbones in all reported metabolites were in line with the previous reports (Busman et al., 2011; Lattanzio et al., 2012; Nakagawa et al., 2013). As the majority of conjugates contained glucoside and/or malonyl moieties, typical fragmentation patterns of these molecules were also considered in the metabolite characterisation exercise. When standards were available, the retention time, accurate mass and MS/MS spectra were used to identify the metabolite. For metabolites

with no available standards, apart from MS/MS fragmentation patterns that were used for structural elucidation, MetExtract could recognise the *Δ m/z* difference between the monoisotopic [e.g. $M+H]^+$ and non-labelled and fully ^{13}C-labelled metabolite. The difference corresponded to the exact number of C-atoms of the uniformly labelled tracer remaining in the parent chemical backbone. A lower ^{13}C count value from 22 and 24 for HT2 and T2, respectively, denoted metabolites that had undergone cleavage of one or more moieties originally present in their carbon skeleton. This additional information proved very useful in facilitating metabolite annotation.

Another powerful technique was fast polarity switching, only utilised in Study III. While some metabolites preferentially ionise in just one of the two modes, many show different ion species in negative mode from the positive, yielding different adducts and in-source fragments. Most HT2 and T2 metabolites were detected in both polarities, except from T2-α-Glc, 3Ac-T2 and feruloyl-T2, which were only detected in the positive ionisation mode, and T2-triol-glucoside, only recognised in the negative mode. Hence, fast polarity switching enabled the detection of those compounds that may not have been "visible" in either negative or positive mode and it facilitated the annotation of ion species by providing complementary information from each mode. It also assisted in significantly minimising the analysis times, as each sample was only run once. Kluger et al. (2014), also applied fast polarity switching to examine the metabolism of $^{13}C_9$-phenylalanine in wheat cell suspension cultures in the presence of DON, during which the Exactive Plus Orbitrap MS was configured, similarly to Study III, to switch the ionisation polarity after each MS scan, which lasted approximately half a second. Importantly, MetExtract was capable of automatically processing fast polarity switching data, by performing feature pair detection separately for each mode and then convoluting the different pairs of the same metabolite conjointly for the positive and negative ionisation mode results.

Altogether, LC–HRMS raw data processing with MetExtract software proved to be very effective in recognising distinct mirror symmetric isotopologue patterns of native and partially labelled modified mycotoxins, filtering out irrelevant biological signals and background noise, and thus enabling the holistic detection of truly relevant biotransformation products that were descendants of the tracer substances under investigation. This analytical workflow has also been successfully utilised to study DON metabolism in wheat (Kluger et al., 2015b) and HT2/T2 in oats (Meng-Reiterer et al., 2016), and has led to the identification of several novel modified mycotoxins. Nevertheless, apart from its numerous benefits, there are also some drawbacks associated with this workflow. The workflow in its entirety is very complex to perform and expensive, as it requires modern LC–HRMS instruments, pure tracer substances with high isotopic enrichment, which are also very costly to produce, and experienced multidisciplinary personnel. In view of these limitations, as fragmentation data are essential for structural elucidation, metabolomics libraries have already been created with

data acquired from commercially available or synthesised standards (Schrimpe-Rutledge et al., 2016). In the future, easy accessibility of researchers to comprehensive fragmentation pattern spectral databases of precursor mycotoxins and common conjugated moieties could substantially support the annotation of novel modified mycotoxins and mapping of metabolic pathways.

On the other hand, a simpler and more pragmatic approach for most mycotoxin research laboratories in the world today, is the application of targeted metabolomics that aim to identify, and quantify when standards are readily available, unpredicted metabolites or expected metabolites such as those typically encountered in toxicokinetics/metabolism studies. Hence, the utilisation of software packages such as MetaboLynx XS, which was used in Study V to investigate the metabolism of trichothecenes in brewing yeast, can be advantageous. MetaboLynx XS is a post-acquisition raw data processing tool that compares LC–HRMS full scan data between control and treatment samples and detects compounds that could be attributed to metabolic transformations. MetaboLynx XS data processing of wort samples spiked with 10,000 µg/L DON, HT2 or T2 during a four-day fermentation by A15 lager yeast, resulted in the putative identification of six metabolites (DON3Glc, 3/15Ac-DON, HT2-sulfate, T2/3-acetyl-HT2 and 3Ac-T2). The proposed metabolites were analysed by LC–QTOF–MS/MS measurements for further confirmation. MetaboLynx XS has been recently applied to study the metabolism of *Fusarium* mycotoxins and modified forms, e.g., the *in vivo* and *in vitro* metabolism of T2 (Yang et al., 2013) and ZEN (Yang et al., 2017), or the de-glucosylation of ZEN14Glc *in vivo* and in human liver microsomes (Yang et al., 2018).

Finally, in addition to the importance of MS analysis and data processing, chromatographic separation also played an important role in uncovering isomeric modified mycotoxins. In order to recognise potential additional isomers of the HT2 and T2 metabolites present, a longer gradient method was employed to achieve chromatographic separation. As a result, with the longer gradient, the EICs of 15-acetyl-T2-tetraol-malonyl-glucoside and HT2-malonyl-glucoside revealed two peaks, one major and one smaller, that were attributed to isomers resulting from the conjugation of malonic acid to different hydroxyl groups of glucose. Likewise, hydroxy-HT2-glucoside and hydroxy-HT2-malonyl-glucoside were detected in the form of three isomers, one major and two smaller peaks, suggesting that either the additional –OH was introduced to different positions of the HT2 backbone or that, again, the malonic acid was attached to different –OH groups of the glucose moiety. In the case of feruloyl-T2 and di-glucosylated HT2 isomers, two peaks were detected with the original chromatographic gradient having slightly different retention times, as was reported for the latter in Lattanzio et al. (2012). Interestingly, the long gradient method revealed that the retention time of the glucosylated T2 metabolite (22.2 min), only detected in barley (Study III), differed from that of the analytical standard T2-α-Glc (21.4 min). The difference in retention times indicates that T2 is probably converted into T2-3-*O*-β-glucoside in barley. These findings

contradict McCormick et al. (2015), who reported the occurrence of T2-α-Glc in oats and barley.

6.1.4 Quantitative determination

In this work, the simultaneous quantification of a group of *Fusarium* mycotoxins and modified forms thereof was exclusively performed with LC–MS/MS-based methods in either SRM or full scan modes. Absolute quantitation was achieved using standard calibrants if present, whereas for the novel modified mycotoxins of Studies III and IV, relative quantification was performed. It is worth mentioning that indirect modified mycotoxin determination was not applied in any of the methods used herein, but they were always directly quantified or semi-quantified.

In Study I, the LC–MS conditions in the TLX–LC–HRMS technique were based on a previously developed and validated method for the determination in cereals and feed matrices of the *Fusarium* mycotoxins DON, ZEN, fumonisins (B_1 and B_2), HT2 and T2 (Ates et al., 2013), which was later extended for the analysis of plant metabolites (Ates et al., 2014). Thus, method development primarily emphasised the appropriate selection of the chromatographic column between a reversed phase C_{18}, a polymeric Cyclone-P and an MCX 2 column. The MCX 2 column was eventually chosen, as it offered sufficient retention and optimal peak shapes for the analytes DON and DON3Glc; the total analysis run time was also shortened to 14.1 min. For the LC–ESI–TQ–MS/MS method, a similar column assessment took place between a HILIC and an amide UPLC column. The latter provided optimal analyte separation, which was found not possible with the former, and it was thus chosen for the inter-laboratory method comparison study. Instruments operating in SRM mode, such as the one utilised here, are capable of yielding some of the lowest analyte LOD values, but it is crucial to undergo proper precursor and product ion selection, as well as vigorous optimisation of MS/MS parameters to achieve the best signal intensities. Therefore, thorough optimisation was carried out during the LC–ESI–TQ–MS/MS method development for the selection of the optimal ionisation mode (ESI^+/ESI^-) per analyte, the selection of quantifier/qualifier ions and for the fine-tuning of the MS/MS conditions. The selected mass transitions and quantifier/qualifier ion ratios for all target analytes were confirmed to be identical in neat standard solution and in matrix. Quantification of DON and DON3Glc was achieved by comparing the peak areas of the samples with those from the external matrix-assisted calibration of barley and wheat.

The same LC–ESI–TQ–MS/MS instrument was also used in Study II, with an Atlantis T3 UPLC column and a matching Atlantis T3 pre-column this time around. As in the previous study, method development was initiated by infusing individual analyte solutions into the MS, operating in both positive and negative ESI modes, for proper precursor and product ion selection. ZEN and most of its derivatives, with the exception

of ZEN14Glc and ZEN16Glc, as well as most of the type B trichothecene derivatives, were more easily ionised in the negative mode, whereas type A trichothecenes showed higher intensities in the ESI positive mode. Following instrument tuning with the target analytes, the chromatographic parameters were developed, focusing on adjustments of the gradient elution programme to accomplish sufficient retention, baseline separation of isomeric pairs and optimal peak shapes for all analytes. One characteristic example of the importance of chromatographic parameters and, in this case, gradient elution optimisation occurred between the modified mycotoxins ZEN14Glc, ZEN16Glc and β-ZEL14Glc which all shared a common precursor ion *m/z* 498.4. The three analytes also produced the same quantifier product ion 498.4>319.1, but β-ZEL14Glc had a different qualifier ion (*m/z* 275.1), which allowed its unique identification among the three compounds. However, the isomeric compounds ZEN14Glc and ZEN16Glc, not only produced the same precursor/product ion (quantifier, qualifier) pairs (*m/z* 498.4>319.1, 283.2) but also chromatographically co-eluted, making them indistinguishable. Hence, a short isocratic elution phase between the 7–9 min of the analytical run was introduced, into the linear gradient programme, which resolved the issue by allowing the identification of these compounds according to their retention times. For quantification, and to compensate for the observed SSE phenomena, matrix-assisted calibration of analyte-free representative sample material was necessary to reduce these effects to some degree and improve the accuracy and sensitivity of the method.

Analyte quantification and relative quantification in Studies III and IV relied on LC–HRMS instrumentation (LC–QTOF–MS and LC–Orbitrap). Absolute quantification was only possible for a handful of HT2 and T2 derived metabolites for which analytical standards were available at the time of the experiments (HT2, HT2-3-Glc, T2, 3Ac-T2 and T2-α-Glc). For all other metabolites, relative quantification was performed to establish their kinetic profiles from EIC integrated peak areas, corrected for the individual ear weight. Lastly, in Study V, an LC–ESI–TQ–MS/MS-based method was used to measure the analyte concentrations in wort and yeast biomass. Again, due to severe matrix effects being observed for DON (SSE = 78% in wort) and DON3Glc (SSE = 53% in wort), matrix-assisted calibration curves were used for quantification. However, multi-mycotoxin methods are nowadays increasingly being developed using stable isotope dilution approaches to address the issue of matrix effects and allow for uncompromised determination of multiple analytes in a variety of matrices (Varga et al., 2012; Zhang et al., 2017; Habler and Rychlik, 2016). Although the utilisation of ^{13}C-internal standards is the best way to dynamically account for matrix effects, matrix-assisted calibration has proven a viable alternative whenever isotopologic standards are not available (Li et al., 2019).

6.2 Natural occurrence of (modified) *Fusarium* mycotoxins in Finnish cereals

The results of the survey revealed that DON was the most abundant analyte and present at the highest concentrations. In the central and eastern areas of Finland, where maximal DON contamination was detected, frequent rainfall occurred between the end of June and beginning of July, according to the Finnish Meteorological Institute (http://www.fmi.fi), a period when the flowering of oats generally takes place. The rainy period was succeeded by warm climatic conditions, often in excess of 25 °C, which are optimal for DON-producing fungal species (Ramirez et al., 2006; Kokkonen et al., 2010; Wegulo, 2012). Until recently, the dominant species in Finland was *F. culmorum* in barley and wheat. However, in the past years, it has gradually been replaced by *F. graminearum*, which is a more prolific DON producer (Yli-Mattila, 2010). Similar fungal population shifts have been documented during the past couple of decades in other northern European countries, including the Netherlands (Waalwijk et al., 2003), the UK (Nicholson et al., 2003), Sweden (Fredlund et al., 2008) and Denmark (Nielsen et al., 2011), to name a few.

The DON levels in Study II were unusually high, reaching levels of up to 23,800 µg/kg in oats and 5510 µg/kg in wheat, compared to survey data collected by the annual Finnish grain quality monitoring programme for several years before and after the 2013 harvest. Importantly, the majority of samples contaminated with the highest DON levels were not intended for human consumption, but for feed (see Subsection 5.2.2). Analytical data generated with a reference method and presented in the grain quality annual report verified these unusually high DON concentrations from samples originating from the same areas (Hietaniemi et al., 2014). According to that report, only 73% of the analysed grains in 2013 fulfilled the requirements for use as food, whereas in the previous year, 92% did. It is noteworthy that sampling of grains in 2013 was mainly targeted at high-risk areas. On rare occasions, similarly elevated DON concentrations have been documented in the literature during the past decade. For instance, about 40% of durum wheat samples collected in northern Italy in 2010 were found to exceed the respective ML for unprocessed cereals destined for human consumption, and the year after, two durum wheat samples were found to be contaminated with more than 25,000 µg/kg DON (Bertuzzi et al., 2014).

From the other native mycotoxins, NIV (incidence 63%) was the second most abundant following DON. It is mainly produced by *F. poae* and in smaller amounts by *F. culmorum* and *F. graminearum* in northern areas (Bottalico and Perrone, 2002) and in Finland (Yli-Mattila et al., 2008). The principally low levels of NIV in samples with high DON and 3Ac-DON presence suggest that *F. culmorum* and *F. graminearum* were predominantly of the 3Ac-DON-producing chemotype IA (Yli-Mattila, 2010). The highest levels of HT2 and T2 were detected in samples from the southern parts of the country

that had a drier and warmer climate than in the rest of the country. Such climatic conditions typically enhance the proliferation of *F. sporotrichioides* and *F. langsethiae* (Kokkonen et al., 2010; Medina and Magan, 2011), the major HT2/T2 producers in Nordic oats (Parikka et al., 2012). Interestingly, a larger proportion of all three types of cereals analysed in Study II were contaminated with HT2 in comparison to T2, and at a greater average concentration. Kokkonen et al. (2010), demonstrated that higher temperatures (25 °C) resulted in increased HT2 production compared to T2 for both *F. sporotrichioides* and *F. langsethiae*, whereas the opposite occurred at lower temperatures (15 °C). Moreover, plants are able to rapidly biotransform T2 into HT2, as shown in Studies III and IV, a fact that could also explain the higher incidence and mean HT2 levels observed in the survey samples. Lastly, ZEN, which is known to be produced at the end of the growing period before harvesting, was measured at low to medium levels of contamination. This was presumably due to the weather being warm and dry at that time in Finland, conditions not favourable for its production (Matthäus et al., 2004).

The availability of analytical standards for all the modified mycotoxins included in the LC–MS/MS method of Study II allowed for the collection of accurate quantitative data that would not otherwise have been possible. The entirety of modified mycotoxins analysed, namely, DON3Glc, HT2-3-Glc, NIV3Glc, ZEN14Glc, ZEN14Sulf, ZEN16Glc, α-ZEL, α-ZEL14Glc, β-ZEL and β-ZEL14Glc, were detected in at least one sample and at concentrations higher than their respective LOQ value. Notably, the natural presence of modified forms such as ZEN16Glc and NIV3Glc in certain types of grains was reported in Study II for the first time ever.

Quantitative measurements with LC–MS/MS revealed that DON3Glc was not only the most prevalent modified mycotoxin, with a staggering 81% incidence in total, but the second most detected analyte overall. The maximum concentration of DON3Glc was 6600 μg/kg in a highly DON-contaminated oat sample intended for feed, but levels of up to 19,000 DON3Glc μg/kg have been documented in barley malt (Habler and Rychlik, 2016). The relative proportion of modified to native mycotoxin (DON3Glc/DON), expressed as a molar ratio, was determined to be between 15–40%, and in a few barley samples reached 65–90%. In EFSA's scientific opinion concerning the risks related to DON and its modified forms, it was stated that from data on a wide variety of cereals, 3Ac-DON, 15Ac-DON and DON3Glc are currently the most relevant modified forms of the mycotoxin in Europe, and their concentration ratios relative to DON were 10%, 15% and 20% to DON, respectively (EFSA, 2017). In the same opinion, it was mentioned that in the category of malt and beer, maximum concentrations for DON3Glc approached those of DON, a fact that might be explained by the formation of DON3Glc during germination and brewing. The highest concentration of NIV3Glc was found in barley (65.3 μg/kg), where this modified mycotoxin was detected in 62% of the samples, in contrast to only 16% in oats (max. 58.3 μg/kg) and 10% in wheat (max. 33.6 μg/kg). Interestingly, the incidence of the precursor NIV among barley and oats was around 70%, but the

former had a significantly lower average concentration (96.6 µg/kg) compared to the latter (635 µg/kg). These figures suggest that barley glucosylated NIV much more efficiently than the other cereal grains. In total, NIV3Glc was present in 31% of the samples and accounted for approximately 5–25% of the total NIV. This modified/native relative proportion range is in agreement with an earlier study reporting that more than 15% of NIV was converted into NIV3Glc in wheat grain artificially infected with *Fusarium* fungi (Nakagawa et al., 2011). Another paper from Japan estimated that the percentage of NIV3Glc relative to NIV was between 12–27% in naturally contaminated wheat (Yoshinari et al., 2014).

The existence of mono- and di-glucosides of HT2 and T2 had been reported a number of times in the literature prior to this work in barley, wheat, oats, corn and various cereal-based products (e.g. Busman et al., 2011; Veprikova et al., 2012; Lattanzio et al., 2012). The molar ratio between HT2-3-Glc to HT2 was in the range of 15–40% and the maximum HT2-3-Glc concentration was measured at 300 µg/kg in an oat sample. Lattanzio et al. (2015) analysed barley samples from Northern Italy and estimated the relative proportion of modified/native HT2-3-Glc/HT2 to be roughly 37% and measured a maximum HT2-3-Glc concentration of 163 µg/kg. The mono-glucosyl congener of T2 was detected in only a few samples and at low levels of not more than 14.5 µg/kg. Remarkably, in the same survey, the sum of HT2 and T2 glucosides ranged between 2% and 280% in relation to the summed concentrations of their precursors. These broad variations can be ascribed to differences in sampling time, cultivars, growing conditions and harvest season, among other factors. Finally, both phase I and II ZEN metabolites were determined in all three types of cereal grains. ZEN producers can also form α- and β-ZEL, which may be further biotransformed by plants and fungi (e.g. species of *Rhizopus* and *Aspergillus*; Brodehl et al., 2014) into conjugated phase II metabolites. It is thus crucial to investigate whether similar biotransformation may occur during the biotechnological production of fermented foods using microorganisms capable of forming α-ZEL and its conjugates. According to the survey findings, ZEN, α- and β-ZEL, along with one or more glucosylated forms and the sulfate conjugate, were found to co-exist. ZEN14Sulf was generally the modified ZEN mycotoxin with the highest abundance with concentrations up to 220 µg/kg. Remarkably, the recently characterised ZEN16Glc was detected in more samples than the well-known ZEN14Glc. A survey study of cereals and cereal-derived food from the Belgian market also reported wide co-occurrence of ZEN with several of its derivatives (De Boevre et al., 2013). More specifically, α- and β-ZEL accounted for 58% and 21% of ZEN, respectively, whereas ZEN14Glc represented an additional 42% and α- and β-ZEL14Glc accounted for 20%; ZEN14Sulf only amounted to about 5% of ZEN. Conducting survey studies to monitor the quality of the raw grains is crucial for economic and food safety reasons. To achieve this goal, analytical standards are urgently needed, as well as more data on the occurrence of modified mycotoxins in food and feed to better characterise the risks associated with these compounds.

6.3 Metabolic fate of trichothecenes

6.3.1 Metabolism by plants

Plants are equipped with an arsenal of natural mechanisms to counteract *Fusarium* infections and reduce mycotoxin accumulation (reviewed in Boutigny et al., 2008). Biotransformation and successional compartmentation are among the most effective plant defences against toxic xenobiotics. The complete inventory of metabolites tentatively identified in Studies III and IV is presented in Tables 6–8. All metabolites detected in the HT2-treated samples were found in the cereal ears inoculated with T2 due to the rapid conversion of T2 into HT2 and subsequent common metabolism. Hence, HT2 is a major metabolite of T2 in barley and wheat, as is for many other organisms (Li et al., 2011).

There is ample scientific evidence that the most common type of *in planta* phase II biotransformation of *Fusarium* mycotoxins is glucosylation. Conjugation of fusariotoxins to glucose molecule(s) by plants has been already described numerous times in the literature for DON, NIV and ZEN, as well as for HT2 and T2. Here, glucosylation was also confirmed to be the principal metabolic reaction. As a matter of fact, all of the HT2 metabolites detected in barley had glucoside moieties incorporated to their structures, and all except two acetylated congeners of HT2 in wheat. Unsurprisingly, glucosylated metabolites also formed the bulk of annotated structures in the T2-treated cereals. Most trichothecenes, however, possess multiple functional groups that could seemingly undergo direct conjugation without necessitating phase I metabolism. In the first ever report describing the existence of HT2 and T2 glucosides, it was suggested that although T2 has only one likely site at C-3 for glucoside attachment, HT2 has an additional location for glucoside conjugation with its free hydroxyl functionality at C-4 (Busman et al., 2011). Lattanzio et al. (2012) also hypothesised that HT2 could be glucosylated at C-3 or C-4 positions, after observing two distinct peaks in naturally contaminated oat and wheat samples that were characterised as mono-glucosylations. A few years later, two HT2 mono-glucoside isomers were measured in barley from Northern Italy, tentatively identified as products of C-3 and C-4 glucose conjugations (Lattanzio et al., 2015). Nevertheless, in Studies III and IV, only a single peak was detected and identified as HT2-3-Glc by HRMS/MS fragmentation and analytical standard measurements in both barley and wheat. The fact that no two peaks were found in Studies III and IV, may be potentially explained by differences in cultivation/experimental conditions or by differences in chromatographic parameters, including column temperature, mobile phase composition and gradient programme. T2-α-Glc was only detected in barley and not in any of the wheat varieties.

From a recent study by Li et al. (2015), it was demonstrated that transgenic wheat, expressing a barley UDP-GT with the ability to rapidly metabolise DON to the less toxic DON3Glc, was significantly more resistant to the spread of FHB compared to the non-transformed controls. Although at present there is a lack of data to confirm or refute an analogous conjecture for HT2 and T2 glucosides, a reduction in their phytotoxic potential is not unreasonable to assume. Furthermore, two isomers tentatively annotated as HT2-di-glucoside were found in both cereals. As the mono-glucosylation of HT2 occurred exclusively at C-3, the two sugar molecules are presumably conjugated at the same position in tandem but by a different enzyme, leading to β-1,4 or β-1,6 di-glucosides. No di-glucosylated metabolites were detected for T2. Despite the absence of such T2 derivatives in these experiments, di-glucosylated T2 has previously been reported in corn (Nakagawa et al., 2013). It is worth mentioning that Zachariasova et al. (2012) described the occurrence of not only di-glucoside, but also tri- and tetra-glucosides of DON in cereal-based products.

The toxicity of trichothecenes, as extensively documented in the literature, is considerably abated if the acetyl group at C-4 is transformed to a hydroxyl group and even more so when both acetoxy moieties from C-4 and C-15 are cleaved (Wu et al., 2013). In the $^{12}C/^{13}C$ experiments, it was revealed that wheat was not only able to de-acetylate T2 into HT2, but also to acetylate it. As previously described, HT2 has two hydroxyl groups at C-3 and C-4 positions, readily susceptible to acetylation, which could potentially result in the formation of either 3-acetyl-HT2 or T2. A feature pair recognised by MetExtract with *m/z* 489.2083 (^{12}C)/511.2833 (^{13}C) indicated the presence of 22 tracer-derived carbon atoms and fitted the sum formula of the ^{13}C-HT2 backbone having a non-labelled acetyl group attached to it. Chromatographically, the retention time of the metabolite was close to but did not precisely match that of T2 (16.2 vs. 16.0 min; Study IV), and it is thus assumed that acetylation most likely occurred at C-3, forming 3-acetyl-HT2. The concentration of this metabolite accounted for less than 0.4% of the applied HT2. In any event, acetylated HT2 was demonstrated to have been formed by wheat, and whether T2 might also have co-existed remains unclear on the basis of available data. Meng-Reiterer et al. (2016), however, confirmed that C-4 acetylation of HT2 to T2 undoubtedly occurred in oats. Nonetheless, de-acetylation of T2 in both barley and wheat was seemingly far faster and more extensive compared to reverse acetylation of HT2, so the potential formation of 3-acetyl-HT2 or T2 after metabolisation of HT2 in wheat was only marginal.

The majority of the other metabolites were derived from HT2-3-Glc by further modifications to its chemical backbone (Figure 14). For instance, malonyl-glucoside congeners are expected to have been formed by conjugation of malonic acid to the glucose moiety, as has been previously shown for ZEN in *Arabidopsis thaliana* (Berthiller et al., 2006) and DON in wheat (Kluger et al., 2015b). Malonyl-glucoside metabolites of alternariol and alternariol-9-*O*-monomethyl ether were also found in suspension cultures

of tobacco BY-2 cells (Hildebrand et al., 2015). In that study, it was shown by NMR data that linkage of the malonyl group can either happen at the C-6 position of the glucose, forming 6-*O*-malonyl-β-D-glucopyranosyl, or at the glucose C-4 position, forming 4-*O*-malonyl-β-D-glucopyranosyl. Thus, NMR analysis of the detected malonyl-glucosides in Studies III and IV would be necessary for exact structure elucidation. Other glucoside-based metabolites putatively identified were the following: 15-acetyl-T2-tetraol-glucoside (loss of isovaleryl moiety at C-8), 15-acetyl-T2-tetraol-malonyl-glucoside (malonyl conjugation and loss of isovaleryl moiety at C-8), 15-acetyl-T2-tetraol-glucoside[–2H] (loss of isovaleryl moiety and keto group formation at C-8; no MS/MS spectrum obtained), hydroxy-HT2-glucoside (hydroxylation at C-3'), hydroxy-HT2-malonyl-glucoside (malonyl conjugation and hydroxylation at C-3'), T2-triol-glucoside (loss of acetyl group at C-15) and dehydro-HT2-glucoside (loss of water from the isovaleryl moiety of hydroxy-HT2-glucoside). The fact that no un-conjugated 15-acetyl-T2-tetraol, T2-triol, hydroxy-HT2 or dehydro-HT2 were detected in the samples indicates that the phase I metabolic reactions hydrolysis, de-acetylation and oxidation eventuated after the conjugation of glucose and malonyl moieties to active functional groups on the HT2 backbone. As the glucosylation of DON has been confirmed to be an important detoxification process in plants (Poppenberger et al., 2003), it is suggested that glucosylated HT2 and T2 are potentially also less phytotoxic than their precursors. In contrast to other organisms, the toxicity of HT2 and T2 is equivalent to plants, but removal of the isovaleryl group of C-8 considerably alleviates their phytotoxic effects (Nishiuchi et al., 2006). Therefore, a single mycotoxin may undergo several detoxification processes *in planta* prior to vacuole compartmentation.

3Ac-T2 and two isomeric derivatives of feruloyl-T2 (tentatively identified as *trans*-feruloyl-T2 and *trans*-isoferuloyl-T2) were the only T2-specific modified forms in barley and wheat bearing the intact skeleton of the toxin. Notably, the conjugation of acetic and ferulic acids occurred directly to T2 without previous hydrolysis of the C-4 acetyl group and formation of HT2. Since ferulic acid naturally exists in plants as *trans*-ferulic acid (Sosulski et al., 1982), the second isomer is more likely *trans*-isoferuloyl-T2. However, the presence of *cis*-feruloyl-T2 should not be entirely excluded, because *cis* ferulic acid can derived from the *trans* form after light-induced non-enzymatic isomerisation (Katase, 1981). Feruloyl conjugates have never previously been reported in the literature, not only for HT2 and T2 but for any other mycotoxin. This constitutes a major finding involving a novel pathway in the detoxification of type A trichothecenes. The study conducted for HT2 and T2 in oats, based on the same ^{13}C-assisted approach, also reported acetic acid and ferulic acid conjugates of T2, together with many glycosylated and hydroxylated metabolites in common with those reported here, and other novel ones such as HT2-malyl-glucoside (Meng-Reiterer et al., 2016). Hardly any malonic acid conjugations for HT2 and T2 were detected in oats, apart from HT2-malonyl-glucoside, which was present at minute concentrations approaching the LOD. Hence, it appears that

malonyltransferases of barley and wheat are either more efficiently expressed compared to oats or they are more active under the tested conditions.

Trichothecene de-epoxidation of the C-12,13 epoxide ring, which confers toxicity to these molecules (Eriksen et al., 2004), is a crucial detoxification process in mammalian metabolism, for which the microflora of the digestive tract is responsible (Karlovsky, 1999). The epoxide group remained intact and unconjugated in all tentatively annotated metabolites of Studies III and IV. In fact, there is experimental evidence that loss of the C-12,13 epoxide ring in type A trichothecenes generally increases phytotoxic activity (Abbas et al., 2013). Additionally, whilst the epoxide ring of type A trichothecenes appears to have little effect on plants, complete detoxification of these mycotoxins to render them safe for humans and animals would require edible plants to be genetically equipped with the ability to remove the epoxide ring (Desjardins and Proctor, 2007). However, de-epoxidation of trichothecenes has not yet been reported *in planta*. In another experiment utilising an identical study design to investigate the metabolism of DON in wheat, GSH conjugation was reported as one of the major metabolic routes together with glucosylation (Kluger et al., 2015a). The authors proposed that GSH either formed a Michael adduct at C-10 or GSH conjugation resulted in the opening of the epoxide ring after nucleophilic attack of the SH group to the C-12 atom of DON. If the latter hypothesis is proven by NMR analysis, it could be worth further exploring as an alternative approach to inactivate the toxicologically relevant epoxide ring of trichothecenes and produce modified mycotoxins with reduced mammalian toxicity, as GSH conjugations of epoxides are likely to be irreversible (Berthiller et al., 2013). Herein, no conjugations to GSH and/or sulfur-containing HT2/T2 metabolites such as glutathione S-cysteinyl-glycine or S-cysteine were identified. Another significant difference between HT2/T2 and DON tested was that no di-glucosylation of DON was detected, although in other studies, multiple glucosylations of DON have been documented in cereals (Zachariasova et al., 2012). Therefore, toxin structure and physiological aspects such cereal varieties, cultivation conditions and the severity of fungal infections can strongly influence the outcome of different experiments.

Time course kinetics of quantified HT2, T2 and HT2-3-Glc for barley and wheat (Remus and CM-82036) are depicted in Figure 13. In both cereal types, T2 was efficiently hydrolysed to HT2 and, in turn, roughly half of it was conjugated into HT2-3-Glc within a day, regardless of whether it was directly applied HT2 or a product of T2 metabolism. Indeed, rapid *in planta* biotransformation of T2 into HT2 has previously been demonstrated for a number of cereals, including maize, wheat, oats and barley, with different conversion rates (Lattanzio et al., 2009). From the wheat experiments (Study IV), which incorporated a more frequent sampling schedule, it was already evident that HT2-3-Glc was the major metabolite 6 h after exposure. This metabolite reached its maximum after between one day and one week, followed by a clear downward trend denoting further metabolism. Meng-Reiterer et al. (2016), reported that oats were more efficient in

this regard, displaying the highest turnover from HT2 and T2 into HT2-3-Glc of the three cereal plants. Taking a closer look at the biotransformation of HT2 metabolites over time, hydroxy-HT2-glucoside, hydroxy-HT2-malonyl-glucoside, HT2-di-glucoside, HT2-malonyl-glucoside and T2-triol-glucoside reached their maximal relative abundances at the ripening timepoint, making them the most relevant for food/feed safety. Due to the lack of analytical standards for these compounds their absolute amount could not be determined, but from comparison of the EIC peak areas, HT2-malonyl-glucoside can be classified as a major metabolite. 3Ac-T2 and ferulyol-T2 attained their highest levels at day one, supporting the assumption that besides glucosylation, alternative metabolic plant responses were activated without necessitating hydrolysis of T2 to HT2. The decline of 3Ac-T2 could potentially be attributed to the reverse reaction back to T2 (de-acetylation) and the decrease of ferulyol-T2 may have been linked to the incorporation of this modified mycotoxin into the plant cell wall (Iiyama et al., 1994; Meng-Reiterer et al., 2016). Only minor deviations were identified between the susceptible (Remus) and resistant (CM-82036) to FHB wheat lines, with no marked disparities being observed in their kinetics profiles. In summary, barley and wheat metabolised HT2 and T2 with typical phase I and II reactions, but the latter were faster and more dominant.

6.3.2 Metabolism by yeast

In Study V, HT2, T2, DON and DON3Glc did not generally interfere with the growth and viability, sugar utilisation or alcohol production of A15 lager yeast, even at levels much higher than normally found in naturally contaminated wort. In particular, the yeast cells were able to tolerate HT2 and DON up to 10,000 µg/L, DON3Glc at 400 µg/L and the mixture of HT2 and T2 (5000 µg/L each) throughout the 96-h fermentation. Resistance of yeast to DON has also been documented in a study by Boeira et al. (1999), who observed no significant inhibition of growth in lager and ale *S. cerevisiae* strains after 24 h, at concentrations approximating those commonly found in naturally contaminated grains (100–2000 µg/L). In the same experiments, significant inhibition of growth only occurred at 100,000 µg/L and inhibition of viability at 200,000 µg/L DON after only 6 h of exposure. Similar adverse effects in cell numbers, dry mass and total protein have been documented in a 24-h fermentation of *S. cerevisiae* treated with 50,000 µg/L DON (Whitehead and Flannigan, 1989). These toxin amounts, however, were much greater than those that the A15 yeast was exposed to in Study V. T2 in the high-dose group was the only toxin that appeared to slightly influence viability in comparison to controls (96% vs. 99.5%), but alcohol production was not negatively affected. This mycotoxin is known to cause a temporary reduction in the rate of yeast fermentation at high concentrations, possibly due to its inhibitory effects on mitochondrial function by hindering oxygen utilisation and causing slowed log phase growth (Wolf-Hall, 2007). In this experiment,

apart from the slightly reduced yeast viability, no similar severe effects were recorded at levels of up to 10,000 µg/L T2.

Overall, faster and more efficient toxin reduction was noted in the samples spiked with 100 µg/L HT2 or T2 compared to samples spiked with 10,000 µg/L. At the low dosing level, there was an immediate decrease (5–13%) in the studied compounds within the first 4 h of fermentation, which could be ascribed to binding of the toxins to the cell walls of yeast, a rapid process that reaches saturation relatively fast (Pfliegler et al., 2015). Based on the chemical composition of yeast cell walls, it is reasonable to assume that this structural layer presents innumerable sites for physical adsorption of molecules such as mycotoxins (Shetty and Jespersen, 2006). Nevertheless, after the initial 24 h, clear differences in the kinetic profiles between type A and type B trichothecenes were seen. At the last sample collection point (96 h), 66% and 69% of the initially dosed HT2 and T2, respectively, were determined in wort, whereas about 85% of DON was measured at the end of the study; a similar adsorption of 11.6% DON by *S. cerevisiae* has been reported (Campagnollo et al., 2015). DON3Glc followed identical kinetic behaviour to DON. It is worth mentioning that in a paper by Scott et al. (1992), DON remained completely intact during a 9-day fermentation by 3 strains of *S. cerevisiae*. Altogether, the findings of Study V are in agreement with most past experiments of similar or longer fermentation durations, showing considerable reductions in trichothecene levels, but nonetheless having high residual mycotoxin amounts at termination (Garda et al., 2005; Inoue et al., 2013). Differences between the present results and those of other publications in the literature could be related to the yeast strains used, toxin concentrations, incubation length and conditions, as well as changes in the physico-chemical properties of mycotoxins (polarity, chemical structure, dissociation constant, etc.). All these parameters can influence the binding, adsorption and metabolism of mycotoxins (Boeira et al., 1999; Faucet-Marquis et al., 2014).

A seminal finding of this study was that no DON was found in the DON3Glc group, indicating that de-conjugation of the glucoside moiety did not occur during fermentation. As a matter of fact, A15 yeast was able to glucosylate DON to DON3Glc, which was confirmed with analytical standard measurements. Traces of DON3Glc were detected in samples collected at the last two time points (72 and 96 h). This was further supported by the detection of small toxin amounts in the yeast biomass, also indicating the uptake of mycotoxins into the cells and biotransformation. Several other papers have documented glucosylation of mycotoxins by fungi as a means of detoxification, e.g., HT2 to HT2-3-Glc and T2 to T2-Glc (Busman et al., 2011; McCormick et al., 2012) or DON to DON3Glc (Tian et al., 2016). Optimisation of the culture conditions to intentionally enhance glucosylation of mycotoxins during brewing fermentation could prove to be a promising mitigation strategy, as according to current knowledge, trichothecene glucosides are considered less toxic than their precursors (Broekaert et al., 2015; Section 2.3).

Nevertheless, acetylation was the dominant biotransformation taking place. A plethora of microorganisms, alongside both trichothecene- and non-trichothecene-producing fungi, are equipped with acetyltransferases that can convert *Fusarium* trichothecenes into less toxic C-3 acetyl derivatives (McCormick et al., 2011). This strategy is employed during the biosynthesis of trichothecenes by mycotoxin-producing organisms for self-protection (Kimura et al., 1998), and is possibly one of the main reasons for the high resistance of yeast to trichothecenes. Indeed, for all three mycotoxins (DON, HT2 and T2), acetylated metabolites were putatively identified by MetaboLynx XS and follow-up LC–MS/MS measurements (detailed MS/MS spectra in Study V), although it was not possible to discern the exact location of the acetylation site for DON (C-3 or C-15) or HT2 (C-3 or C-4). Consequently, acetylation of HT2 may have produced T2 or 3-acetyl-HT2, or even both, as was previously discussed for Study IV. Acetylation of T2 could only have occurred at C-3, as this is the only available site on its chemical backbone accessible for direct acetylation. As in plants and other organisms, yeast was also capable of de-acetylating T2 by hydrolysis at C-4, leading to the formation of HT2 (Studies III and IV; EFSA, 2011). This type of detoxification reaction was only detected in the T2 high-dose group, and the conversion only accounted for 0.2% of the initially dosed toxin with no further de-acetylated metabolites such as T2-triol being detected.

Lastly, a novel modified mycotoxin was putatively identified as HT2-sulfate. It was initially captured by MetaboLynx XS as its sodium adduct (*m/z* 527.1536, $[M+Na]^+$) matched the theoretical accurate *m/z* value of the metabolite in question. Follow-up LC–MS/MS analysis revealed a fragment ion corresponding to the $[M+H]^+$ precursor of HT2 and two of its characteristic fragments. This metabolite was only present in samples collected after 72 h in the high-exposure HT2 group, and at such low concentrations that it became very challenging to obtain a proper LC–MS/MS spectrum (presented in Study V). It also appeared to have a shorter chromatographic retention time compared to HT2 (10.43 vs. 12.85 min), indicating higher hydrophilicity that would be expected from a polar metabolite. A number of sulfate conjugates of mycotoxins have been characterized in fungi and plants, including ZEN14Sulf (Engelhardt et al., 1988; El-Sharkaway et al., 1991; Plasencia and Mirocha, 1991; Berthiller et al., 2006), DON-3-sulfate, DON-15-sulfate (Warth et al., 2015), alternariol-3-sulfate, alternariol-9-sulfate and alternariol-9-O-methyl ether-3-sulfate (Soukup et al., 2016).

6.4 Methodological considerations

In spite of tremendous advances in analytical sciences and the existence of methods able to simultaneously determine hundreds of contaminants, each measurement is still the outcome of a series of compromises, starting from sampling all the way to quantification.

Analysis of mycotoxins and modified forms constitutes a major challenge because of the broad variability in their physico-chemical properties, as well as due to the heterogeneity and complexity of matrices from which they have to be extracted, preventing a one-size-fits-all approach (Shephard, 2016). Additionally, mycotoxins and their derivatives are typically encountered at relatively low levels in most samples, thus further complicating matters. Modern advanced analytical techniques, despite these complications, can facilitate determinations with high selectivity and sensitivity and permit retrospective identification of non-target analytes (Anfossi et al., 2016).

A challenge for most LC–MS/MS-based methods, including those developed and used herein, is unequivocally sample preparation and specifically analyte extraction. Efficient extraction, with its importance previously highlighted in Subsections 2.2.1 and 6.1.1, is largely dependent on analyte chemical structures and their compatibility with the extraction solution applied. As a consequence, insufficient extraction of modified mycotoxins from the matrices analysed may have hindered the detection of certain forms. Although brief trials to select the most appropriate extraction solutions were conducted in all the studies, more intensive investigations to optimise the balance between minimal matrix interference and maximum analyte recovery may have yielded superior results (Zhou et al., 2012). Bound non-extractable mycotoxins, incorporated/attached to plant or yeast cellular macromolecules, also elude determination (Berthiller et al., 2013). The absence of any dedicated clean-up procedure in all metabolomics studies in order to attenuate analyte losses could have rendered undetectable certain low intensity metabolite ion signals due to matrix effects (Krska et al., 2008). Underestimation of modified mycotoxin species/content may also have occurred to some extent in the survey study, as more modified mycotoxins could have been present for which no analytical standards were available at the time of measurement.

One important drawback of any type of metabolomics experiment is the reporting of false positives, i.e. incorrect hits. In the SIL-based experiments, identification of metabolites based on the distinct Δ *m/z* difference between monoisotopic and ^{13}C-labelled isotopologues from HRMS data may have led to the detection of false positives, e.g. due to inaccurate pairings of artefacts, isotope and complex adducts peaks (Brown et al., 2011). Thus, to significantly reduce the number of false positives, the isotope pattern of both native and labelled metabolite ions and chromatographic co-elution are taken into account. Inevitably, a few false positives were picked up by MetExtract from data generated in Studies III and IV. These were manually excluded from further processing based on certain criteria such as higher than expected $^{12}C/^{13}C$ mass shifts than the number of C atoms contained in the intact toxins, or implausible isotopologue intensity ratios. Furthermore, LC–HRMS/MS spectra that did not contain any HT2 and T2 characteristic fragment ions were also disregarded. It should be mentioned that considering the multiplicity of substances often present in biological samples, in this case barley and wheat ears, and without having applied any sort of sample clean-up, a very

low rate of false positives was observed. This alone demonstrates the exceptionally high selectivity and effectiveness of the utilised SIL-assisted metabolomics approach.

In addition, because of methodological limitations (e.g. LOD of the analytical methods) and the strict selection criteria applied to MetExtract software, in an attempt to reduce the incidence of false positives, some metabolites with a low abundance may have remained undetected. To some extent, the high degree of ^{13}C-isotopic enrichment of the precursor toxins, resulting in the absence of M'–1 isotopologues of the labelled metabolites, could have contributed to this. Hence, MetExtract hits detected from even a single replicate were investigated in order to avoid having any lost metabolites. In Study III, there was an issue with the automated identification of 15-acetyl-T2-tetraol-based metabolites in the T2-treated barley samples because of the unexpectedly high $^{12}C/^{13}C$ signal ratios. This irregularity was attributed to an impurity in the T2 inoculation solution, which after purity assessment was found to contain 3% non-labelled neosolaniol. This impurity was then probably transformed into 15-acetyl-T2-tetraol by de-acetylation at C-4 from the plants, enhancing the ^{12}C-mass signal and hampering its detection. Emphasis thus should be given to the use of tracer toxins of the highest possible purity when conducting state-of-the-art metabolomics experiments to achieve optimal performance and accurate results. It should be noted that prior to the application of SIL-assisted metabolomics in mycotoxin research, the only modified HT2 and T2 mycotoxins identified were their glucosylated forms, which shows that apart from a few unavoidable limitations, this experimental framework is a very powerful tool and even more so in its latest iteration (Bueschl et al., 2017), offering tremendous possibilities to "unmask" modified mycotoxins *in planta*. Lastly, in Study V, where only targeted metabolite screening was performed, additional unpredicted modified forms could have been formed by yeast and were never detected.

Although all modified mycotoxins reported in Studies II–IV are assumed to have been produced as a result of plant metabolism, given the fact that fungi are autonomously capable of their biosynthesis, their true origin cannot be definitively ascertained, and some metabolites may have been (partially) synthesised by indigenous fungi residing on plants. Examples of common phase II metabolic processes between plants and fungi are glucosylation, acetylation and sulfation (Freire and Sant'Ana, 2017). From a food/feed safety perspective, what actually matters is not the origin of toxins but the type and content. Importantly, the modified *Fusarium* mycotoxins reported in this thesis are not exhaustive and additional modifications may be materialised by different cereal varieties. Thus, the assembly of all available literature is necessary to fully describe the *in planta* metabolism of every mycotoxin. Again, annotated metabolites in Studies III–V for which no analytical standard was available should be considered as putative until NMR analyses are performed. NMR measurements, however, were outside the scope of this work.

6.5 Outlook

The risks associated with the presence of mycotoxins in food and feed have prompted many countries of the developed world to implement modern agricultural practices and set in place legislatively regulated food processing and marketing systems, which have greatly reduced mycotoxin exposure in these populations (Milićević et al., 2010). In the past few decades, however, mycotoxin production and associated problems with crop quality and safety have undeniably been affected by the manifold implications of climate change and other human activities (Paterson and Lima, 2010; Parikka et al., 2012). Literature data suggest that global warming is having a profound effect on cereal cultivation, and this phenomenon has a large impact on plant–pathogen interactions, causing changes in host physiology and morphology (Chakraborty et al., 2000), earlier flowering (Van der Fels-Klerx et al., 2012; Olesen et al., 2012) and reduced yields (Lesk et al., 2016). The geographical distribution of mycotoxigenic fungi is also adjusting to the new conditions, and there are reasons to believe that the composition of these species and pre-harvest levels of mycotoxins will change (Yli-Mattila, 2010; Wu et al., 2011). As a consequence, alterations in the fungal infection patterns of staple crops will arise, leading to unique mycotoxin contamination profiles and, in turn, the formation of novel modified mycotoxins with unpredictable toxicological manifestations. A constantly evolving mycotoxin landscape begets enormous challenges for routine surveillance programmes and compels researchers to devise high throughput, robust and accurate analytical approaches for monitoring mycotoxin contamination.

Methods that can accommodate a wide range of chemistries for the concurrent determination of precursor and modified forms, and which are capable of structurally elucidating novel modified mycotoxins, are essential for ensuring the safety of food- and feedstuffs (Krska et al., 2017). The downside of direct approaches is the requirement for analytical standards for quantification, which are seldom available for modified mycotoxins. The synthesis of modified mycotoxin standards with the aspiration to become commercially accessible will bolster research related to their formation, toxicity, stability and fate along the manufacturing and food chains. Nevertheless, whenever analytical standards are not readily available, indirect quantification by conversion of conjugated mycotoxins to their parents through chemical or enzymatic hydrolysis can be a useful alternative (Cirlini et al., 2012). As far as their formation is concerned, SIL-assisted metabolomics in combination with computational LC–HRMS data processing embodies a powerful workflow for identifying novel modified mycotoxins, and it would be ideal to exploit its utilisation for all major mycotoxins. Having said that, such experiments are costly, time-consuming and require a high level of multidisciplinary expertise and resources. In this context, computational modelling to predict the metabolism of mycotoxins could become valuable. Although still in its infancy, predictive modelling

based on a combination of functional genomics and ecological data would empower researchers to understand the transcriptomic responses of fungi to climatic changes and predict the progression of fungal infections and mycotoxin production (Magan et al., 2011; Gilbert et al., 2016). *In silico* tools can also be used to predict plant–pathogen interactions and the resulting modified mycotoxins based on the type of crops, environmental conditions and expected fungal populations (e.g. Steinmetz et al., 2009).

Human exposure to mycotoxins and modified derivatives may arise from the consumption of contaminated edible crops and from carryover of these compounds to animal-derived food. According to occurrence surveys, the ubiquitous presence of single modified mycotoxins in plants and processed foods can frequently reach 30% of the precursor form, and on occasion even surpass it. An aggregate summation of all mycotoxin derivatives present in the diet is anticipated to contribute a significantly higher (modified) mycotoxin burden. The worldwide tendencies to use oats and other cereals as meat substitutes and to promote the intake of fibre-rich foods as part of a healthy diet may increase exposure to mycotoxins for certain groups of the population that heavily rely on plant-based foods to fulfil their daily dietary requirements. Therefore, exposure data used by regulatory authorities for risk assessment may warrant re-evaluation to cover new food consumption scenarios.

At the same time, it is of outmost importance to investigate the ADME properties and inherent toxicological effects of modified mycotoxins in order to devise appropriate risk management measures. To date, the toxicological relevance of modified *Fusarium* mycotoxins for food and feed safety has remained largely obscure. Adequate information has only been generated for a select few well-studied derivatives of DON and ZEN; for some other modified mycotoxins, scarce toxicity data can also be found in the literature. Even so, the general consensus is that modified mycotoxins as a whole are as yet poorly studied from a toxicological perspective and significant data gaps exist. The governing perception of the scientific community is that DON3Glc, the most toxicologically examined modified mycotoxin, can be regarded as a detoxification product with reduced bioavailability and toxicological activity in living organisms (Gratz, 2017). However, the release of parent toxins along the mammalian GIT (e.g. ZEN14Sulf) may pose a threat to human and animal health, albeit largely dependent on the digestive phase in which it may take place (De Boevre et al., 2015). Early de-conjugation might exert toxicity on the upper digestive system and renders toxic aglycones more prone to intestinal absorption and in turn, increased systemic uptake. As digestion progresses and chyme reaches the large intestine, the absorption of toxins decreases dramatically, with local toxicity to intestinal epithelium and disturbances to microbial flora constituting the major risks (Nathanail et al., 2016). The relevance of novel modified forms to food and feed safety require thorough assessment with targeted toxicity testing, primarily focusing on those modified mycotoxins that are expected to be present at high concentrations in edible plants at harvest and not on those appearing only as

intermediates during cultivation or degraded on the field. This last point alone, embodies a fundamental justification why metabolomics studies, such as the ones performed in this Ph.D. thesis, are pivotal for guiding toxicologists to test the most relevant compounds.

From a legislative perspective, modified mycotoxins are currently not covered by any regulatory framework in the EU. The potential risks associated with these substances have been under evaluation by EFSA during the past years, with a number of scientific opinions already published (EFSA, 2014; EFSA, 2016; EFSA, 2017a; EFSA, 2017b; EFSA, 2017c), and are now in the pipeline of the EU legislation (Dall'Asta and Berthiller, 2016). According to the CONTAM Panel of EFSA, the presence of modified forms is considered a non-negligible additional risk, assuming the complete release of aglycones from conjugated metabolites. As an example, more than 30 modified forms of ZEN have been described in the literature, and the establishment of a group-TDI for certain mycotoxins and their modified derivatives in food and feed thus seems inevitable. To estimate the risk to animal and human health of parent *Fusarium* mycotoxins together with their modified forms, EFSA assessed them together and established a group-TDI for the sum of these compounds and estimated the cumulative risk of exposure to them. Even though this is a pragmatic approach, and some of the values are based on experimental data (e.g. uterotrophic activity of certain ZEN modified forms), the majority all these suppositions may add significant ambiguity in the risk assessment. There are several parameters that could contribute to a high overall uncertainty, including exposure estimates, the lack of proper validation of analytical methods, limited toxicological data and assignment of inappropriate relative potency factors, which according to EFSA are more likely to over- than under-estimate the risk. Precautionary safety approaches, however, without the support of sound risk-benefit analyses, may not be practically applicable, resulting in unnecessary, unfavourable risk assessments with potentially destructive repercussions on trade, food/feed producers and distributors worldwide (Dellafiora and Dall'Asta, 2016). Therefore, coordinated actions at an international level to prioritise and address all sources of uncertainty via efficient resource allocation and collaborations between academia, research organisations and regulatory authorities is the future of food/feed safety to ensure consumer protection from mycotoxins and toxic derivatives based on pragmatic approaches and reliable exposure and toxicity data.

7 CONCLUSIONS

The research carried out in Studies I–V revolved around modified *Fusarium* mycotoxins produced by plants and fungi and generated fundamental information concerning their formation, natural occurrence and metabolic fate. Quantitative LC–MS/MS analytical techniques for rapid, accurate and sensitive determination of *Fusarium* mycotoxins and modified derivatives, as well as state-of-the-art qualitative methodology allowing for the annotation and identification of novel modified forms, were developed and applied.

In Study I, an inter-laboratory comparison was conducted between online sample clean-up and four conventional sample preparation techniques for the analysis of DON and its major modified form, DON3Glc, in barley and wheat. The automated sample clean-up (TurboFlow chromatography), together with extract and shoot, were the most user-friendly approaches and offered the best all-around performance in accordance with legislative criteria. On the other hand, the MycoSep 227 clean-up column yielded satisfactory performance for DON but was unable to accommodate the more polar DON3Glc, even with an additional acetonitrile elution step. Likewise, inadequate results were obtained with centrifugal filtration, which did not achieve sufficiently low LOQ values for the target analytes and required an additional evaporation-reconstitution step that may not be compatible with unstable analytes. Overall, on-line clean-up showed high potential for use in both research and routine analytical applications for analytes with varying physico-chemical properties.

A multi-mycotoxin LC–MS/MS method was developed and validated to investigate the natural occurrence of *Fusarium* mycotoxins and modified forms in Finnish barley, oats and wheat grains of the 2013 harvest (Study II). Data gathered from this survey revealed the presence of a multitude of modified mycotoxins, of which DON3Glc was the most abundant, followed by HT2-3-Glc and NIV3Glc. This was the first report of modified mycotoxin contamination in Finland, and the presence of some of the analytes (e.g. ZEN16Glc and NIV3Glc) was documented for the first time worldwide in one or more of the crops analysed. Of the precursor mycotoxins, DON, NIV and HT2 were most prevalent and present at the highest concentrations, especially in oats. The very high contamination levels of DON can be attributed to favourable climatic conditions for *Fusarium* mycotoxin production, the emergence of more prolific DON-producing fungal species, as a result of climatic changes, and due to the fact that sampling was concentrated in high risk areas.

According to the findings from the SIL-assisted *in planta* untargeted metabolomics (Studies III and IV), barley and wheat extensively biotransformed HT2 and T2 to polar metabolites by employing both phase I and II reactions. There was a considerable overlap between HT2 and T2 metabolites between the two cereal grains, because of the rapid conversion of a substantial proportion of T2 into HT2, which was

further metabolised into several common novel modified mycotoxins. Metabolic processes involved the hydrolysis of acetyl and isovaleryl groups, hydroxylation, as well as covalent binding of glucose, malonic acid, acetic acid and ferulic acid. Kinetic data revealed that HT2-3-Glc was the major modified metabolite for both mycotoxins, already reaching its maximum abundance one day after toxin treatment, and was it eventually metabolised into different glucosylated derivatives. The qualitative data evaluation utilising SIL toxin tracers, LC–HRMS instrumentation and MetExtract software proved to be a powerful experimental setup to unravel *in planta* HT2 and T2 metabolism and identify modified mycotoxins potentially relevant to food and feed safety.

Lastly, in the brewing fermentation (Study V), *S. pastorianus* A15 lager yeast displayed high tolerance to HT2, T2, DON and DON3Glc at concentrations far in excess of those occurring in naturally contaminated wort. After 4 days of fermentation, yeast was able to reduce the initially dosed mycotoxin levels by 15% to 34% via physical binding and/or metabolisation. LC–MS/MS data revealed the formation of glucose and sulfate conjugates along with (de)-acetylation metabolic products. Most importantly, DON3Glc remained stable under the experimental conditions and did not revert back to its parent form. The fundamental significance of these results to the brewing industry is that the influence of mycotoxins on yeast appeared to be less relevant than the impact of yeast on the concentration and composition of mycotoxins in beer. Therefore, brewing fermentation bears great potential for augmentation as an effective mitigation strategy, as long as derivatives with reduced toxicity and bioavailability emerge and concomitantly preserve their stability during brewing and digestion.

As a closing statement, and in an attempt to address the cover page question, whether modified *Fusarium* mycotoxins pose a threat in disguise to human and/or animal safety –in the view of the author– this query must be split into two parts. On the one hand, analytical methodologies and experimental setups relying on targeted/untargeted metabolomics, such as those developed and presented in this dissertation, proved more than competent in unveiling previously unknown "disguised" modified mycotoxins and deciphering the metabolic pathways involved. On the other, without compound-specific toxicological data for the vast majority of modified mycotoxins, the risks (threat) associated with them still remain largely uncertain. For now, the primary focus of research activities and risk management measures should remain on precursor mycotoxins, which are among the most noxious food and feed contaminants, causing adverse effects in humans and animals in addition to colossal economic and food losses worldwide. However, if mycotoxins could become efficiently converted into chemically stable and toxicologically inert substances, in the field or during food manufacturing, modified *Fusarium* mycotoxins could be then deemed as a precious ally (instead of a threat) for food and feed safety and as catalyst for accomplishing global food security.

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